



**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
REQUEST FOR FILING APPLICATION UNDER 37 CFR 53**

For: Commissioner of Patents and Trademarks
Washington, D.C. 20231

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Date: **September 29, 1997**

A/Box Sequence

This is a request for filing a new **Continuation-in-Part** PATENT APPLICATION under Rule 53 entitled:
SEED COAT SPECIFIC DNA REGULATORY REGION AND PEROXIDASE
without a filing fee and/or without an executed inventor's oath/declaration.

This application is made by the below identified inventor(s). Attached hereto are the following papers:

- ☒ An abstract together with
57 pages of specification and claims including
29 numbered claims and also attached is/are
16 sheets of accompanying drawings.
☐ This application is based on the following prior foreign application(s):

Application No.	Country	Filing Date
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respectively, and priority is hereby claimed therefrom.

- ☐ This application is based on the following prior provisional application(s):
- | Application No. | Filing Date |
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respectively, and priority is hereby claimed therefrom.

- ☐ Certified copy/ies of foreign applications attached.
☒ This application is a ☐ continuation/☐ division/☒ continuation-in-part of application
Serial No. **08/723,414**, filed **September 30, 1996**.
☒ Please amend the specification by inserting before the first line:
-- This application is a ☐ continuation/☐ division/☒ continuation-in-part of application
Serial No. **08/723,414**, filed **September 30, 1996**.
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application No. , filed .
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Provisional Application No. , filed .
☐ Preliminary amendment to claims (attached hereto), to be entered before calculation of the fee.
☒ Also attached. Hard Copy and Paper Copy of Sequence Listing

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U.S. PATENT APPLICATION

Inventor(s): Mark GIJZEN

Invention: SEED COAT SPECIFIC DNA REGULATORY REGION AND
PEROXIDASE

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SPECIFICATION

SEED COAT SPECIFIC DNA REGULATORY REGION AND PEROXIDASE

The present invention relates to a novel DNA molecule comprising a plant seed coat specific DNA regulatory region and a novel structural gene encoding a peroxidase. The seed-coat specific DNA regulatory region may also be used to control the expression of other genes of interest within the seed coat.

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BACKGROUND OF THE INVENTION

Full citations for references appear at the end of the Examples section.

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Peroxidases are enzymes catalyzing oxidative reactions that use H_2O_2 as an electron acceptor. These enzymes are widespread and occur ubiquitously in plants as isozymes that may be distinguished by their isoelectric points. Plant peroxidases contribute to the structural integrity of cell walls by functioning in lignin biosynthesis and suberization, and by forming covalent cross-linkages between extension, cellulose, pectin and other cell wall constituents (Campa, 1991). Peroxidases are also associated with plant defence responses and resistance to pathogens (Bowles, 1990; Moerschbacher 1992). Soybeans contain 3 anionic isozymes of peroxidase with a minimum M_r of 37 kDa (Sessa and Anderson, 1981). Recently one peroxidase isozyme, localised within the seed coat of soybean, has been characterized with a M_r of 37 kDa (Gillikin and Graham, 1991).

20

In an analysis of soybean seeds, Buttery and Buzzell (1968) showed that the amount of peroxidase activity present in seed coats may vary substantially among different cultivars. The presence of a single dominant gene *Ep* causes a high seed coat peroxidase phenotype (Buzzell and Buttery, 1969). Homozygous recessive *epep* plants are ~100-fold lower in seed coat peroxidase activity. This results from a reduction in the amount of peroxidase enzyme present, primarily in the hourglass cells of the subepidermis (Gijzen *et al.*, 1993). In plants carrying the *Ep* gene, peroxidase is heavily concentrated in the hourglass cells (osteosclereids). These cells form a highly differentiated cell layer with thick, elongated secondary walls and large intercellular spaces (Baker *et al.*, 1987). Hourglass cells develop between the epidermal macrosclereids and the underlying articulated parenchyma, and are a prominent feature of seed coat anatomy at full maturity. The cytoplasm exudes from the hourglass cells upon imbibition with water and a distinct peroxidase isozyme constitutes five to 10% of the total soluble protein in *EpEp* seed coats. It is not known why the hourglass cells accumulate large amounts of peroxidase, but the sheer abundance and relative purity of the enzyme in soybean seed coats is significant because peroxidases are versatile enzymes with many commercial and industrial applications. Studies of soybean seed coat peroxidase have shown this enzyme to have useful catalytic properties and a high degree of thermal stability even at extremes of pH (McEldoon *et al.*, 1995). These properties result in the preferred use of soybean peroxidase, over that of horseradish peroxidase, in diagnostic assays as an enzyme label for antigens, antibodies, oligonucleotide probes, and within staining techniques. Johnson et al report on the use of soybean peroxidase for the deinking of printed waste paper (U.S. 5,270,770;

December 6, 1994) and for the biocatalytic oxidation of primary alcohols (U.S. 5,391,488; February 13, 1996). Soybean peroxidase has also been used as a replacement for chlorine in the pulp and paper industry, or as formaldehyde replacement (Freiberg, 1995).

5 An anionic soybean peroxidase from seed coats has been purified (Gillikin and Graham, 1991). This protein has a pI of 4.1 and M_r of 37 kDa. A method for the bulk extraction of peroxidase from seed hulls of soybean using a freeze thaw technique has also been reported (U.S. 5,491,085, February 13, 1996, Pokara and Johnson).

10 Lagrimini et al (1987) disclose the cloning of a ubiquitous anionic peroxidase in tobacco encoding a protein of M_r of 36 kDa. This peroxidase has also been over expressed in transgenic tobacco plants (Lagrimini et al 1990) and Maliyakal discloses the expression of this gene in cotton (WO 95/08914).

15 Huangpu et al (1995) reported the partial cloning of a soybean anionic seed coat peroxidase. The 1031 bp sequence contained an open reading frame of 849 bp encoding a 283 amino acid protein with a M_r of 30,577. The M_r of this peroxidase is 7 kDa less than what one would expect for a soybean seed coat peroxidase as reported by Gillikin and Graham (1991) and possibly represents another peroxidase isozyme
20 within the seed coat.

The upstream promoter sequences for two poplar peroxidases have been described by Osakabe et al (1995). A number of characteristic regulatory sites were identified from comparison of these sequences to existing promoter elements. Additionally, a cryptic promoter with apparent specificity for seed coat tissues was isolated from tobacco by a promoter trapping strategy (Fobert et al. 1994). The upstream regulatory sequences associated with the Ep gene in soybean are distinct from these and other previously characterized promoters. The soybean Ep promoter drives high-level expression in a cell and tissue specific manner. The peroxidase protein encoded by the Ep gene accumulates in the seed coat tissues, especially in the hour glass cells of the subepidermis. Minimal expression of the gene is detected in root tissues.

One problem arising from the desired use of soybean seed coat peroxidase is that there is variability between soybean varieties regarding peroxidase production (Buttery and Buzzell, 1986; Freiberg, 1995). Due to the commercial interest in the use of soybean seed coat peroxidase new methods of producing this enzyme are required. Therefore, the gene responsible for the expression of the 37 kDa isozyme in soybean seed coat was isolated and characterized.

Furthermore, novel regulatory regions obtained from the genomic DNA of soybean seed coat peroxidase have been isolated and characterized and are useful in directing the expression of genes of interest in seed coat tissues.

SUMMARY OF THE INVENTION

The present invention relates to a DNA molecule that encodes a soybean seed coat peroxidase and associated DNA regulatory regions.

This invention also embraces isolated DNA molecules comprising the nucleotide
5 sequence of either SEQ ID NO:1 (the cDNA encoding soybean seed coat peroxidase)
SEQ ID No:2 (the genomic sequence).

This invention also provides for a chimeric DNA molecule comprising a seed
coat-specific regulatory region having nucleotides 1-1532 of SEQ ID NO:2 and a gene
10 of interest under control of this DNA regulatory region. Also included within this
invention are chimeric DNA molecules comprising genomic DNA sequences
exemplified by nucleotides 412-1041, 1234-2263 or 2430-2691 of SEQ ID NO:2.
Furthermore, this invention is directed to isolated DNA molecules comprising at least

- 1) 24 contiguous nucleotides selected from nucleotides 1-1532 of SEQ ID
15 NO:2;
- 2) 32 contiguous nucleotides selected from nucleotides 412-1041 of SEQ
ID NO:2;
- 3) 23 contiguous nucleotides selected from nucleotides 1234-2263 of SEQ
ID NO:2; or
- 20 4) 22 contiguous nucleotides selected from nucleotides 2430-2691 of SEQ
ID NO:2.

The present invention also provides for vectors which comprise DNA molecules encoding soybean seed coat peroxidase. Such a construct may include the DNA regulatory region from SEQ ID NO:2, including nucleotides 1-1532, or at least 24 contiguous nucleotides selected from nucleotides 1-1532 of SEQ ID NO:2 in conjunction with the seed coat peroxidase gene, or the seed coat peroxidase gene under
5 the control of any suitable constitutive or inducible promoter of interest.

This invention is also directed towards vectors which comprise a gene of interest placed under the control of a DNA regulatory element derived from the genomic sequence encoding soybean seed coat peroxidase. Such a regulatory element
10 includes nucleotides 1-1532 of SEQ ID NO:2, or at least 24 contiguous nucleotides selected from nucleotides 1-1532 of SEQ ID NO:2. Elements comprising nucleotides 412-1041, 1234-2263 or 2430-2691 of SEQ ID NO:2, or 32 contiguous nucleotides selected from nucleotides 412-1041 of SEQ ID NO:2, 23 contiguous nucleotides selected from nucleotides 1234-2263 of SEQ ID NO:2, or 22 contiguous nucleotides
15 selected from nucleotides 2430-2691 of SEQ ID NO:2 may also be used.

This invention also embraces prokaryotic and eukaryotic cells comprising the vectors identified above. Such cells may include bacterial, insect, mammalian, and plant cell cultures.

20

This invention also provides for transgenic plants comprising the seed coat peroxidase gene under control of constitutive or inducible promoters. Furthermore,

December 6, 1994) and for the biocatalytic oxidation of primary alcohols (U.S. 5,391,488; February 13, 1996). Soybean peroxidase has also been used as a replacement for chlorine in the pulp and paper industry, or as formaldehyde replacement (Freiberg, 1995).

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20 within the seed coat.

this invention also relates to transgenic plants comprising the DNA regulatory regions of nucleotides 1-1532 of SEQ ID NO:2 controlling a gene of interest, or comprising genes of interest in functional association with genomic DNA sequences exemplified by nucleotides 412-1041, 1234-2263 or 2430-2691 of SEQ ID NO:2. Also embraced by this invention are transgenic plants having regulatory regions comprising at least 24
5 contiguous nucleotides selected from nucleotides 1-1532 of SEQ ID NO:2, 32 contiguous nucleotides selected from nucleotides 412-1041 of SEQ ID NO:2, 23 contiguous nucleotides selected from nucleotides 1234-2263 of SEQ ID NO:2, or 22 contiguous nucleotides selected from nucleotides 2430-2691 of SEQ ID NO:2.

10 This invention is also directed to a method for the production of soybean seed coat peroxidase in a host cell comprising:

- i) transforming the host cell with a vector comprising an oligonucleotide sequence that encodes soybean seed coat peroxidase; and
- ii) culturing the host cell under conditions to allow expression of the
15 soybean seed coat peroxidase.

This invention also provides for a process for producing a heterologous gene of interest within seed coats of a transformed plant, comprising propagating a plant transformed with a vector comprising a gene of interest under the control of
20 nucleotides 1-1532 of SEQ ID NO:2. Furthermore, this invention embraces a process for producing a heterologous gene of interest within seed coats of a transformed plant, comprising propagating a plant transformed with a vector comprising a gene of interest

Although the present invention is exemplified by a soybean seed coat peroxidase and adjacent DNA regulatory regions, in practice any gene of interest can be placed downstream from the DNA regulatory region for seed coat specific expression.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

Figure 1 is the cDNA and deduced amino acid sequence of soybean seed coat peroxidase. Nucleotides are numbered by assigning +1 to the first base of the ATG start codon; amino acids are numbered by assigning +1 to the N-terminal Gln residue after cleavage of the putative signal sequence. The N-terminal signal sequence, the region of the active site, and the heme-binding domain are underlined. The numerals I, II and III placed directly above single nucleotide gaps in the sequence indicate the three intron splice positions. The target site and direction of five different PCR primers are shown with dotted lines above the nucleotide sequence. An asterisk (*) marks the translation stop codon.

Figure 2 is the genomic DNA sequence of the Soybean seed coat peroxidase.

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Figure 3 is a comparison of soybean seed coat peroxidase with other closely related plant peroxidases. The GenBank accession numbers are provided next to the name of the plant from which the peroxidase was isolated. The accession number for the soybean sequence is L78163. (A) A comparison of the nucleic acid sequences; (B) A comparison of the amino acid sequences.

20

Figure 4 is a restriction fragment length polymorphisms between *EpEp* and *epep* genotypes using the seed coat peroxidase cDNA as probe. Genomic DNA of soybean lines OX312 (*epep*) and OX347 (*EpEp*) was digested with restriction enzyme, separated by electrophoresis in a 0.5% agarose gel, transferred to nylon, and hybridized with ³²P-labelled cDNA encoding the seed coat peroxidase. The size of the hybridizing fragments was estimated by comparison to standards and is indicated on the right.

Figure 5 exhibits the structure of the *Ep* Locus. A 17 kb fragment including the *Ep* locus is illustrated schematically. A 3.3 kb portion of the gene is enlarged and exons and introns are represented by shaded and open boxes, respectively. The final enlargement of the 5' region shows the location and DNA sequence around the 87 bp deletion occurring in the *ep* allele of soybean line OX312. Nucleotides are numbered by assigning +1 to the first base of the ATG start codon.

Figure 6 displays PCR analysis of *EpEp* and *epep* genotypes using primers derived from the seed coat peroxidase cDNA. Genomic DNA from soybean lines OX312 (*epep*) and OX347 (*EpEp*) was used as template for PCR analysis with four different primer sets. Amplification products were separated by electrophoresis through a 0.8% agarose gel and visualized under UV light after staining with ethidium bromide. Genotype and primer combinations are

indicated at the top of the figure. The size in base pairs of the amplified DNA fragments are indicated on the right.

5 **Figure 7** exhibits PCR analysis of an F₂ population from a cross of *EpEp* and *epep* genotypes. Genomic DNA was used as template for PCR analysis of the parents (P) and 30 F₂ individuals. The cross was derived from the soybean lines OX312 (*epep*) and OX347 (*EpEp*). Plants were self pollinated and seeds were collected and scored for seed coat peroxidase activity. The symbols (-) and (+) indicate low and high seed coat peroxidase activity, respectively. Primers prx9+ and prx10- were used in the amplification reactions. Products were
10 separated by electrophoresis through a 0.8% agarose gel and visualized under UV light after staining with ethidium bromide. The migration of molecular markers and their corresponding size in kb is also shown (lanes M).

Figure 8 displays PCR analysis of six different soybean cultivars with primers derived
15 from the seed coat peroxidase cDNA sequence. Genomic DNA was used as template for PCR analysis of three *EpEp* cultivars and three *epep* cultivars. Primers used in the amplification reactions and the size of the DNA product is indicated on the left. Products were separated by electrophoresis through a 0.8% agarose gel and visualized under UV light after staining with ethidium
20 bromide.

(A) Forward and reverse primers are downstream from deletion

(B) Forward primer anneals to site within deletion

(C) Primers span deletion

Figure 9 shows the accumulation of peroxidase RNA in tissues of GEp and *epep* plants. **Figure 9(A):** A comparison of peroxidase transcript abundance in cultivars Harosoy 63 (Ep) or Marathon (ep). Seed and pod tissues were sampled at a late stage of development corresponding to a whole seed fresh weight of 250 mg. Root and leaf tissue was from six week old plants. Autoradiograph exposed for 96 h. **Figure 9(B):** Developmental expression of peroxidase in cultivar Harosoy 63 (Ep). Flowers were sampled immediately after opening. Seed coat tissues were sampled at four stages of development corresponding to a whole seed fresh weight of: lane 1, 50 mg; lane 2, 100 mg; lane 3, 200 mg; lane 4, 250 mg. Autoradiograph exposed for 20 h.

DESCRIPTION OF PREFERRED EMBODIMENT

The present invention is directed to a novel oligonucleotide sequence encoding a seed coat peroxidase and associated DNA regulatory regions.

According to the present invention DNA sequences that are "substantially
5 homologous" includes sequences that are identified under conditions of high stringency. "High stringency" refers to Southern hybridization conditions employing washes at 65°C with 0.1 x SSC, 0.5 % SDS.

By "DNA regulatory region" it is meant any region within a genomic sequence
10 that has the property of controlling the expression of a DNA sequence that is operably linked with the regulatory region. Such regulatory regions may include promoter or enhancer regions, and other regulatory elements recognized by one of skill in the art. A segment of the DNA regulatory region is exemplified in this invention, however, as is understood by one of skill in the art, this region may be used as a probe to identify
15 surrounding regions involved in the regulation of adjacent DNA, and such surrounding regions are also included within the scope of this invention.

In the context of this disclosure, the term "promoter" or "promoter region" refers to a sequence of DNA, usually upstream (5') to the coding sequence of a
20 structural gene, which controls the expression of the coding region by providing the recognition for RNA polymerase and/or other factors required for transcription to start at the correct site.

There are generally two types of promoters, inducible and constitutive. An "inducible promoter" is a promoter that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer the DNA sequences or genes will not be transcribed. Typically the protein factor, that binds specifically to an inducible promoter to activate transcription, is present in an inactive form which is then directly or indirectly converted to the active form by the inducer. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, herbicide or phenolic compound or a physiological stress imposed directly by heat, cold, salt, or toxic elements or indirectly through the action of a pathogen or disease agent such as a virus. A plant cell containing an inducible promoter may be exposed to an inducer by externally applying the inducer to the cell or plant such as by spraying, watering, heating or similar methods.

By "constitutive promoter" it is meant a promoter that directs the expression of a gene throughout the various parts of a plant and continuously throughout plant development. Examples of known constitutive promoters include those associated with the CaMV 35S transcript and *Agrobacterium* Ti plasmid nopaline synthase gene.

The chimeric gene constructs of the present invention can further comprise a 3' untranslated region. A 3' untranslated region refers to that portion of a gene comprising a DNA segment that contains a polyadenylation signal and any other regulatory signals capable of effecting mRNA processing or gene expression. The

polyadenylation signal is usually characterized by effecting the addition of polyadenylic acid tracks to the 3' end of the mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5' AATAAA-3' although variations are not uncommon.

5 Examples of suitable 3' regions are the 3' transcribed non-translated regions containing a polyadenylation signal of *Agrobacterium* tumour inducing (Ti) plasmid genes, such as the nopaline synthase (*Nos* gene) and plant genes such as the soybean storage protein genes and the small subunit of the ribulose-1, 5-bisphosphate carboxylase (ssRUBISCO) gene. The 3' untranslated region from the structural gene
10 of the present construct can therefore be used to construct chimeric genes for expression in plants.

 The chimeric gene construct of the present invention can also include further enhancers, either translation or transcription enhancers, as may be required. These
15 enhancer regions are well known to persons skilled in the art, and can include the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence to ensure translation of the entire sequence. The translation control signals and initiation codons can be from a variety of origins, both natural and synthetic. Translational initiation regions may be provided from the
20 source of the transcriptional initiation region, or from the structural gene. The sequence can also be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA.

To aid in identification of transformed plant cells, the constructs of this invention may be further manipulated to include plant selectable markers. Useful selectable markers include enzymes which provide for resistance to an antibiotic such as gentamycin, hygromycin, kanamycin, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as *GUS* (β-glucuronidase), or luminescence, such as luciferase are useful.

Also considered part of this invention are transgenic plants containing the chimeric gene construct of the present invention. Methods of regenerating whole plants from plant cells are known in the art, and the method of obtaining transformed and regenerated plants is not critical to this invention. In general, transformed plant cells are cultured in an appropriate medium, which may contain selective agents such as antibiotics, where selectable markers are used to facilitate identification of transformed plant cells. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be used to establish repetitive generations, either from seeds or using vegetative propagation techniques.

The constructs of the present invention can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-injection, electroporation, etc. For reviews of such techniques see for example

Weissbach and Weissbach (1988) and Geierson and Corey (1988). The present invention further includes a suitable vector comprising the chimeric gene construct.

Buttery and Buzzell (1968) showed that the amount of peroxidase activity present in seed coats may vary substantially among different cultivars. The presence
5 of a single dominant gene *Ep* causes a high seed coat peroxidase phenotype (Buzzell and Buttery, 1969). Homozygous recessive *epep* plants are ~100-fold lower in seed coat peroxidase activity. This results from a reduction in the amount of peroxidase enzyme present, primarily in the hourglass cells of the subepidermis (Gijzen *et al.*, 1993). In plants carrying the *Ep* gene, peroxidase is heavily concentrated in the
10 hourglass cells (osteosclereids). These cells form a highly differentiated cell layer with thick, elongated secondary walls and large intercellular spaces (Baker *et al.*, 1987).

Screening a seed coat cDNA library prepared from *EpEp* plants with a degenerate primer derived from the active site domain of plant peroxidase resulted in
15 a high frequency of positive clones. Many of these clones encode identical cDNA molecules and indicate that the corresponding mRNA is an abundant transcript in developing seed coat tissues. The sequence of the cDNA is shown in Figure 1.

Previous studies on soybean seed coat peroxidase indicated that this enzyme is
20 heavily glycosylated and that carbohydrate contributes 18% of the mass of the apo-enzyme (Gray *et al.*, 1996). The seven potential glycosylation sites identified from the amino acid sequence of the seed coat peroxidase (Figure 1) would accommodate the

five or six N-linked glycosylation sites proposed by Gray *et al.* (1996). The heme-binding domain encompasses residues Asp161 to Phe171 and the acid-base catalysis region from Gly33 to Cys44. The two regions are highly conserved among plant peroxidases and are centred around functional histidine residues, His169 and His40. There are eight conserved cysteine residues in the mature protein that provide for four di-sulfide bridges found in other plant peroxidases and predicted from the crystal structure of peanut peroxidase (Welinder, 1992; Schuller *et al.*, 1996). Other conserved areas include residues Cys91 to Ala105 and Val119 to Leu127 that occur in or around helix D. The most divergent aspects of the seed coat peroxidase protein sequence are the carboxy- and amino-terminal regions. These sequences probably provide special targeting signals for the proper processing and delivery of the peptide chain. It is possible the carboxy-terminal extension of the seed coat peroxidase is removed at maturity, as has been shown for certain barley and horseradish peroxidases (Welinder, 1992).

The molecular mass of the enzyme has been determined by denaturing gel electrophoresis to be 37 kDa (Sessa and Anderson, 1981; Gillikin and Graham, 1991) or 43 kDa (Gijzen *et al.*, 1993). Analysis by mass spectrometry indicated a mass of 40,622 Da for the apo-enzyme and 33,250 Da after deglycosylation (Gray *et al.*, 1996). These values are in good agreement with the mass of 35,377 Da calculated from the predicted amino acid sequence for the mature apo-protein prior to glycosylation and other modifications. Huangpu et al (1995) reported an anionic seed coat peroxidase having a M_r of 30,577 Da and characterized a partial cDNA encoding this protein.

This 1031 bp cDNA contained an open reading frame of 849 bp encoding a 283 amino acid protein. There are several differences between this reported sequence and the sequence of this invention that are manifest at the amino acid level (see Figure 3 for sequence comparison). The enzyme encoded by the gene reported by Huangpu et al is different from that of this invention as the peroxidase of this invention has a M_r of

5 35,377 Da.

Genomic DNA blots probed with the seed coat peroxidase cDNA produced two or three hybridizing fragments of varying intensity with most restriction enzyme digestions, despite that several peroxidase isozymes are present in soybean. The results indicate that this seed coat peroxidase is present as a single gene that does not share sufficient homology with most other peroxidase genes to anneal under conditions of high stringency.

The genomic DNA sequence comprises four exons spanning bp 1533-1752 (exon 1), 2383 -2574 (exon 2), 3605-3769 (exon 3) and 4033-4516 (exon 4) and three introns comprising 1752-2382 (intron 1), 2575-3604 (intron 2) and 3770-4516 (intron 3), of SEQ ID NO:2. Features of the upstream regulatory region of the genomic DNA include a TATA box centred on bp 1487; a cap signal 32 bp down stream centred on bp 1520. Also noted within the genomic sequence are three polyadenylation signals centred on bp 4520, 4598, 4663 and a polyadenylation site at bp 4700.

This promoter is considered seed coat specific since the peroxidase protein encoded by the Ep gene accumulates in the seed coat tissues, especially in the hourglass cells of the subepidermis, and is not expressed in other tissues, aside from a marginal expression of peroxidase in the root tissues. This is also true at the transcriptional level (see Figure 9). The DNA regulatory regions of the genomic sequence of Figure 2 are used to control the expression of the adjacent peroxidase gene in seed coat tissue. Such regulatory regions include nucleotides 1-1532. Other regions of interest include nucleotides 1752-2382, 2575-3604 and/or 3770-4032 of SEQ ID NO:2. Therefore other proteins of interest may be expressed in seed coat tissues by placing a gene capable of expressing the protein of interest under the control of the DNA regulatory elements of this invention. Genes of interest include but are not restricted to herbicide resistant genes, genes encoding viral coat proteins, or genes encoding proteins conferring biological control of pest or pathogens such as an insecticidal protein for example *B. thuringiensis* toxin. Other genes include those capable of the production of proteins that alter the taste of the seed and/or that affect the nutritive value of the soybean.

A modified DNA regulatory sequence may be obtained by introducing changes into the natural sequence. Such modifications can be done through techniques known to one of skill in the art such as site-directed mutagenesis, reducing the length of the regulatory region using endonucleases or exonucleases, increasing the length through the insertion of linkers or other sequences of interest. Reducing the size of DNA regulatory region may be achieved by removing 3' or 5' regions of the regulatory

region of the natural sequence by using an endonuclease such as BAL 31 (Sambrook et al 1989). However, any such DNA regulatory region must still function as a seed coat specific DNA regulatory region.

It may be readily determined if such modified DNA regulatory elements are
5 capable of acting in a seed coat specific manner transforming plant cells with such regulatory elements controlling the expression of a suitable marker gene, culturing these plants and determining the expression of the marker gene within the seed coat as outlined above. One may also analyze the efficacy of DNA regulatory elements by introducing constructs comprising a DNA regulatory element of interest operably
10 linked with an appropriate marker into seed coat tissues by using particle bombardment directed to seed coat tissue and determining the degree of expression of the regulatory region as is known to one of skill in the art.

Two tandemly arranged genes encoding anionic peroxidase expressed in stems
15 of *Populus kitakamiensis*, *prxA3a* and *prxA4a* have been cloned and characterized (Osakabe et al, 1995). Both of these genomic sequences contained four exons and three introns and encoded proteins of 347 and 343 amino acids, respectively. The two genes encode distinct isozymes with deduced M_r s of 33.9 and 34.6 kDa. Furthermore, a 532 bp promoter derived from the peroxidase gene of *Armoracia*
20 *rusticana* has also been reported (Toyobo KK, JP 4,126,088, April 27, 1992). However, a search using GenBank revealed no substantial similarity between the promoter region, or introns 1, 2 and 3 of this invention and those within the literature.

Digestion of the genomic DNA with *Bam*HI or *Sac*I revealed restriction fragment length polymorphisms that distinguished *EpEp* and *epep* genotypes. Although the *Xba*I digestion did not produce a readily detectable polymorphism, the size of the hybridizing fragment in both genotypes was ~14 kb. Thus, a 0.3 kb size difference is outside of the resolving power of the separation for fragments this large. Sequence analysis of *EpEp* and *epep* genotypes indicates that the mutant *ep* allele is missing 87 bp of sequence at the 5' end of the structural gene. This would account for the drastically reduced amounts of peroxidase enzyme present in seed coats of *epep* plants since the deletion includes the translation start codon and the entire N-terminal signal sequence. However, the 87 bp deletion cannot account for the differences observed in the RFLP analysis since the missing fragment does not include a *Bam*HI site and is much smaller than the 0.3 kb polymorphism detected in the *Sac*I digestion. Thus, other genetic rearrangements must occur in the vicinity of the *ep* locus that lead to these polymorphisms.

The results shown here indicate that the mutation causing low seed coat peroxidase activity occurs in the structural gene encoding the enzyme. This mutation is an 87 bp deletion in the 5' region of the gene encompassing the translation start site. Several different low peroxidase cultivars share a similar mutation in the same area, suggesting that the recessive *ep* alleles have a common origin or that the region is prone to spontaneous deletions or rearrangements.

Due to the industrial interest in soybean seed coat peroxidase, alternate sources for the production of this enzyme are needed. The DNA of this invention, encoding the seed coat soybean peroxidase under the control of a suitable promoter and expressed within a host of interest, can be used for the preparation of recombinant soybean seed coat peroxidase enzyme.

5

Soybean seed coat peroxidase has been characterized as a lignin-type peroxidase that has industrially significant properties ie: high activity and stability under acidic conditions; exhibits wide substrate specificity; equivalent catalytic properties to that of *Phanerochaete chrysosporium* lignin peroxidase (the currently preferred enzyme used for treatment of industrial waste waters (Wick 1995) but is at least 150-fold more stable; more stable than horseradish peroxidase which is also used in industrial effluent treatments and medical diagnostic kits (McEldoon *et al.*, 1995). These properties are useful within industrial applications for the degradation of natural aromatic polymers including lignin and coal (McEldoon *et al.*, 1995), and the preferred use of soybean peroxidase, over that of horseradish peroxidase, in medical diagnostic tests as an enzyme label for antigens, antibodies, oligonucleotide probes, and within staining techniques (Wick 1995). Soybean peroxidase is also used in the deinking of printed waste paper (Johnson *et al.*, U.S. 5,270,770; December 6, 1994) and for the biocatalytic oxidation of primary alcohols (Johnson *et al.*, U.S. 5,391,488; February 13, 1996). Soybean peroxidase has also been used as a replacement for chlorine in the pulp and paper industry, in order to remove chlorine, phenolic or aromatic amine containing pollutants from industrial waste waters (Wick 1995), or as formaldehyde

replacement (Freiberg, 1995) for use in adhesives, abrasives, and protective coatings (e.g. varnish and resins, Wick 1995).

Furthermore, the seed coat peroxidase gene may be expressed in an organ or tissue specific manner within a plant. For example, the quality and strength of cotton
5 fiber can be improved through the over-expression of cotton or horseradish peroxidase placed under the control of a fibre-specific promoter (Maliyakal, WO 95/08914; April 6, 1995).

Similarly, seed-specific DNA regulatory regions of this invention may be used
10 to control expression of genes of interest such as:

- i) genes encoding herbicide resistance, or
- ii) biological control of insects or pathogens (e.g. *B. thuringiensis*), or
- iii) viral coat proteins to protect against viral infections, or
- iv) proteins of commercial interest (e.g. pharmaceutical), and
- 15 v) proteins that alter the nutritive value, taste, or processing of seeds

within the seed coat of plants.

While this invention is described in detail with particular reference to preferred embodiments thereof, said embodiments are offered to illustrate but not to limit the
20 invention.

EXAMPLES

Plant material

All soybean (*Glycine max* [L.] Merr) cultivars and breeding lines were from the
5 collection at Agriculture Canada, Harrow, Ontario.

Seed Coat cDNA library Construction and Screening

High seed coat peroxidase (*EpEp*) soybean cultivar Harosoy 63 plants were
10 grown in field plots outdoors. Pods were harvested 35 days after flowering and seeds
in the mid-to-late developmental stage were excised. The average fresh mass was 250
mg per seed. Seed coats were dissected and immediately frozen in liquid nitrogen. The
frozen tissue was lyophilized and total RNA extracted in 100 mM Tris-HCl pH 9.0,
20 mM EDTA, 4% (w/v) sarkosyl, 200 mM NaCl, and 16 mM DTT, and precipitated
15 with LiCl using the standard phenol/chloroform method described by Wang and
Vodkin (1994). The poly(A)⁺ RNA was purified on oligo(dT) cellulose columns prior
to cDNA synthesis, size selection, ligation into the λ ZAP Express vector, and
packaging according to instructions (Stratagene). A degenerate oligonucleotide with the
5' to 3' sequence of TT(C/T)CA(C/T)GA(C/T)TG(C/T)TT(C/T)GT was 5' end
20 labelled to high specific activity and used as a probe to isolate peroxidase cDNA clones
(Sambrook *et al.*, 1989). Duplicate plaque lifts were made to nylon filters (Amersham),
UV fixed, and prehybridized at 36 °C for 3 h in 6 x SSC, 20 mM Na₂HPO₄ (pH6.8),

5 x Denhardt's, 0.4 % SDS, and 500 $\mu\text{g/mL}$ salmon sperm DNA. Hybridization was in the same buffer, without Denhardt's, at 36 °C for 16 h. Filters were washed quickly with several changes of 6 x SSC and 0.1 % SDS, first at room temperature and finally at 40°C, prior to autoradiography for 16 h at -70°C with an intensifying screen.

5 *Genomic DNA Isolation, Library Construction, and DNA Blot Analysis*

Soybean genomic DNA was isolated from leaves of greenhouse grown plants or from etiolated seedlings grown in vermiculite. Plant tissue was frozen in liquid nitrogen and lyophilized before extraction and purification of DNA according to the method of Dellaporta *et al.* (1983). Restriction enzyme digestion of 30 μg DNA, separation on 0.5 % agarose gels and blotting to nylon membranes followed standard protocols (Sambrook *et al.*, 1989). For construction of the genomic library, DNA purified from Harosoy 63 leaf tissue was partially digested with *Bam*HI and ligated into the λ FIX II vector (Stratagene). Gigapack XL packaging extract (Stratagene) was used to select for inserts of 9 to 22 kb. After library amplification, duplicate plaque lifts were hybridized to cDNA probe.

Blots or filter lifts were prehybridized for 2 h at 65°C in 6 x SSC, 5 x Denhardt's, 0.5 % SDS, and 100 $\mu\text{g/mL}$ salmon sperm DNA. Radiolabelled cDNA probe (20 to 50 ng) was prepared using the Ready-to-Go labelling kit (Pharmacia) and ^{32}P -dCTP (Amersham). Unincorporated ^{32}P -dCTP was removed by spin column chromatography before adding radiolabelled cDNA to the hybridization buffer

(identical to prehybridization buffer without Denhardt's). Hybridization was for 20 h at 65°C. Membranes were washed twice for 15 min at room temperature with 2 x SSC, 0.5 % SDS, followed by two 30 min washes at 65°C with 0.1 x SSC, 0.5 % SDS. Autoradiography was for 20 h at -70°C using an intensifying screen and X-OMAT film (Kodak).

5

DNA Sequencing

Sequencing of DNA was performed using dye-labelled terminators and Taq-FS DNA polymerase (Perkin-Elmer). The PCR protocol consisted of 25 cycles of a 30 sec melt at 96°C, 15 sec annealing at 50°C, and 4 min extension at 60°C. Samples were analyzed on an Applied Biosystems 373A Stretch automated DNA sequencer.

10

Polymerase Chain Reaction

PCR amplifications contained 1 ng template DNA, 5 pmol each primer, 1.5 mM MgCl₂, 0.15 mM deoxynucleotide triphosphates mix, 10 mM Tris-HCl, 50 mM KCl, pH 8.3, and 1 unit of Taq polymerase (Gibco BRL) in a total volume of 25 µL. Reactions were performed in a Perkin-Elmer 480 thermal cycler. After an initial 2 min denaturation at 94°C, there were 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 52°C, and 2 min extension at 72°C. A final 7 min extension at 72°C completed the program. The following primers were used for PCR analysis of genomic DNA:

15

20

prx2+ CTTCCAAATATCAACTCAAT
prx6- TAAAGTTGGAAAAGAAAGTA
prx9 ATGCATGCAGGTTTTTCAGT
prx10- TTGCTCGCTTTCTATTGTAT
prx12+ TCTTCGATGCTTCTTTCACC
5 prx29+ CATAACAATACGTACGTGAT

RNA Isolation

For isolation of RNA, tissue was harvested from greenhouse grown plants,
10 dissected, frozen in liquid nitrogen, and lyophilized prior to extraction. Total RNA was
purified from seed coats, embryos, pods, leaves, and flowers using standard
phenol/chloroform method (Sambrook et al., 1989). This method did not afford good
yields of RNA from roots, therefore this tissue was extracted with Triazole reagent
(GibcoBRL) and total RNA purified according to manufacturers' instructions with an
15 additional phenol-chloroform extraction step. The amount of RNA was estimated by
measuring absorbance at 260 and 280 nm, and by electrophoretic separation in
formaldehyde gels followed by staining with ethidium bromide and comparison to
known standards. Total RNA (10 μ g per sample) was prepared, subject to
electrophoresis through a 1% agarose gel containing formaldehyde, and then stained
20 with ethidium bromide to ensure equal loading of samples. The gel was blotted to
nylon (Hybond™N, Amersham) according to standard methods and the RNA was fixed
to the membrane by UV cross linking.

Seed Coat Peroxidase Assays

The F₃ seed was measured for peroxidase activity to score the phenotype of the F₂ population because the seed testa is derived from maternal tissue. The seeds were briefly soaked in water and the seed coat was dissected from the embryo and placed in a vial. Ten drops (~500 µL) of 0.5% guaiacol was added and the sample was left to stand for 10 min before adding one drop (~50 µL) of 0.1% H₂O₂. An immediate change in colour of the solution, from clear to red, indicates a positive result and high seed coat peroxidase activity.

10 **Example 1:** *The Seed Coat Peroxidase cDNA and genomic DNA sequences*

To isolate the seed coat peroxidase transcript, a cDNA library was constructed from developing seed coat tissue of the *EpEp* cultivar Harosoy 63. The primary library contained 10⁶ recombinant plaque forming units and was amplified prior to screening. A degenerate 17-mer oligonucleotide corresponding to the conserved active site domain of plant peroxidases was used to probe the library. In screening 10,000 plaque forming units, 12 positive clones were identified. The cDNA insert size of the clones ranged from 0.5 to 2.5 kb, but six clones shared a common insert size of 1.3 kb. These six clones (*soyprx03*, *soyprx05*, *soyprx06*, *soyprx11*, *soyprx12*, and *soyprx14*) were chosen for further characterization since the 1.3 kb insert size matched the expected peroxidase transcript size. Sequence analysis of the six clones showed that they contained identical cDNA transcripts encoding a peroxidase and that each resulted

from an independent cloning event since the junction between the cloning vector and the transcript was different in all cases.

Since it was not clear that the entire 5' end of the cDNA transcript was complete in any of the cDNA clones isolated, the structural gene corresponding to the seed coat peroxidase was isolated from a Harosoy 63 genomic library. A partial *Bam*HI digest of genomic DNA was used to construct the library and more than 10⁶ plaque forming units were screened using the cDNA probe. A positive clone, G25-2-1-1-1, containing a 17 kb insert was identified and a 4.7 kb region encoding the peroxidase was sequenced SEQ ID NO:2. This region includes 1532 nucleotides of the 5' region of the peroxidase gene.

The genomic sequence matched the cDNA sequence except for three introns encoded within the gene. The genomic sequence also revealed two additional translation start codons, beginning one bp and 10 bp upstream from the 5' end of the longest cDNA transcript isolated. Figure 1 shows the deduced cDNA sequence. The open reading frame of 1056 bp encodes a 352 amino acid protein of 38,106 Da. A heme-binding domain, a peroxidase active site signature sequence, and seven potential N-glycosylation sites were identified from the deduced amino acid sequence. The first 26 amino acid residues conform to a membrane spanning domain. Cleavage of this putative signal sequence releases a mature protein of 326 residues with a mass of 35,377 Da and an estimated pI of 4.4.

Relevant features of the genomic fragment (Figure 2) include four exons at bp 192-411 (exon 1; 1533-1751 of SEQ ID NO:2), 1042 -1233 (exon 2; 2383-2574 of SEQ ID NO:2), 2263-2429 (exon 3; 4033-4516 fo SEQ ID NO:2) and 2692-3174 (exon 4; 1752-2382 of SEQ ID NO:2) and three introns at bp 412-1041 (intron 1; 1752-2382 of SEQ ID NO:2), 1234-2263 (intron 2; 2575-3604 of SEQ ID NO:2) and 2430-2691 (intron 3; 3770-4032 of SEQ ID NO:2). The 1532 bp regulatory region of the genomic DNA include a TATA box centred on bp 1487 and a cap signal 32 bp down stream centred at bp 1520 of SEQ ID NO:2. Also noted within the genomic sequence are three polyadenylation signals centred on bp 4520, 4598, 4700 and a polyadenylation site at bp 4700 of SEQ ID NO:2.

10

Figure 3 illustrates the relationship between the soybean seed coat peroxidase and other selected plant peroxidases. The soybean sequence is most closely related to four peroxidase cDNAs isolated from alfalfa, (see Figure 3) sharing from 65 to 67% identity at the amino acid level with the alfalfa proteins (X90693, X90694, X90692, el-Turk et al 1996; L36156, Abrahams et al 1994). When compared with other plant peroxidases, soybean seed coat peroxidase exhibits from 60 to 65% identity with poplar (D30653 and D30652, Osakabe et al 1994)) and flax (L0554, Omann and Tyson 1995); 50 to 60% identity with horseradish (M37156, Fujiyama et al. 1988), tobacco (D11396, Osakabe et al 1993), and cucumber (M91373, Rasmussen et al. 1992); and 49% identity with barley (L36093, Scott-Craig et al. 1994), wheat (X85228, Baga et al 1995) and tobacco (L02124, Diaz-De-Leon et al 1993) peroxidases.

20

A comparison of the promoter region, 1-1532 of SEQ ID NO:2, indicates that there are no similar sequences present within the GENBANK database.

Example 2: *DNA Blot Analysis Using the Seed Coat Peroxidase cDNA Probe Reveals Restriction Fragment Length Polymorphisms Between EpEp and epep*
5 *Genotypes*

Genomic DNA blots of OX347 (*EpEp*) and OX312 (*epep*) plants were hybridized with ³²P-labelled cDNA to estimate the copy number of the seed coat peroxidase gene and to determine if this locus is polymorphic between the two
10 genotypes. Figure 4 shows the hybridization patterns after digestion with *Bam*HI, *Xba*I, and *Sac*I. Restriction fragment length polymorphisms are clearly visible in the *Bam*HI and *Sac*I digestions. The *Bam*HI digestion produced a strongly hybridizing 17 kb fragment and a faint 3.4 kb fragment in the *EpEp* genotype. The 3.4 kb *Bam*HI fragment is visible in the *epep* genotype but the 17 kb fragment has been replaced by
15 a signal at >20 kb. The *Sac*I digestion resulted in detection of three fragments in *EpEp* and *epep* plants. At least two fragments were expected here since the cDNA sequence has a *Sac*I site within the open reading frame. However, the smallest and most strongly hybridizing of these fragments is 5.2 kb in *EpEp* plants and 4.9 kb in *epep* plants. Digestion with *Xba*I produced hybridizing fragments of ~14 kb and 7.8 kb for both
20 genotypes, with the larger fragment showing a stronger signal.

Example 3: *A Deletion Mutation Occurs in the Recessive ep Locus*

The structural gene encoding the seed coat peroxidase is schematically illustrated in Figure 5. The 17 kb *Bam*HI fragment encompassing the gene includes 191 bp of sequence upstream from the translation start codon, three introns of 631 bp, 1030 bp, and 263 bp, and 13 kb of sequence downstream from the polyadenylation site. The arrangement of four exons and three introns and the placement of introns within the sequence is similar to that described for other plant peroxidases (Simon, 1992; Osakabe *et al.* 1995).

Primers were designed from the DNA sequence to compare *EpEp* and *epep* genotypes by PCR analysis. Figure 6 shows PCR amplification products from four different primer combinations using OX312 (*epep*) and OX347 (*EpEp*) genomic DNA as template. The primer annealing site for prx29+ begins 182 bp upstream from the ATG start codon; the remaining primer sites are shown in Figure 1. Amplification with primers prx2+ and prx6-, and with prx12+ and prx10- produced the expected products of 1.9 kb and 860 bp, respectively, regardless of the *Ep/ep* genotype of the template DNA. However, PCR amplification with primers prx9+ and prx10-, and with prx29+ and prx10- generated the expected products only when template DNA was from plants carrying the dominant *Ep* allele. When template DNA was from an *epep* genotype, no product was detected using primers prx9+ and prx10- and a smaller product was amplified with primers prx29+ and prx10-. The products resulting from amplification of OX312 or OX347 template DNA with primers prx29+ and prx10-

were directly sequenced and compared. The polymorphism is due to an 87 bp deletion occurring within this DNA fragment in OX312 plants, as shown in Figure 5. This deletion begins nine bp upstream from the translation start codon and includes 78 bp of sequence at the 5' end of the open reading frame, including the prx9+ primer annealing site.

5

To test whether this deletion mutation cosegregates with the seed coat peroxidase phenotype, genomic DNA from an F₂ population segregating at the *Ep* locus was amplified using primers prx9+ and prx10- and F₃ seed was tested for seed coat peroxidase activity. Figure 7 shows the results from this analysis. Of the 30 F₂ individuals tested, all 23 that were high in seed coat peroxidase activity produced the expected 860 bp PCR amplification product. The remaining seven F₂'s with low seed coat peroxidase activity produced no detectable PCR amplification products.

Finally, to determine if the OX312(*epep*) and OX347(*EpEp*) breeding lines are representative of soybean cultivars that differ in seed coat peroxidase activity, several cultivars were tested by PCR analysis using primer combinations targeted to the *Ep* locus. Figure 8 shows results from this analysis of six different soybean cultivars, three each of the homozygous dominant *EpEp* and recessive *epep* genotypes. As observed with OX312 and OX347, amplification products of the expected size were produced with primers prx12+ and prx10- regardless of the genotype, whereas *epep* genotypes yielded no product with primers prx9+ and prx10- or a smaller fragment with primers prx29+ and prx10-.

Example 4 Developmental Pattern of Expression of the *Ep* gene

The seed coat peroxidase mRNA levels were determined by hybridizing RNA gel blots with radio labelled cDNA probe. The figure illustrates the transcript abundance in various tissues of *epep* and *EpEp* plants. The mRNA accumulated to high levels in seed coat tissues of *EpEp* plants, especially in the later stages development when whole seed fresh weight exceeded 50 mg. Low levels of transcript could also be detected in root tissues but not in the flower, embryo, pod or leaf. The transcript could also be detected in seed coat and root tissues *epep* plants but in drastically reduced amounts compared to the *EpEp* genotype. The reduced amounts of peroxidase mRNA present in seed coats of *epep* plants indicates that the transcriptional process and/or the stability of the resulting mRNA is severely affected. The *Ep* gene has a TATA box and a 5' cap signal beginning 47 bp and 15 bp, respectively, upstream from the translation start codon. The 87 bp deletion in the *ep* allele extends into the 5' cap signal and therefore could interfere with transcript processing. Regardless, any resulting transcript will not be properly translated since the AUG initiation codon and the entire amino-terminal signal sequence is deleted from the *ep* allele. Not wishing to be bound by theory, the lack of peroxidase accumulation in seed coats of *epep* plants appears to be due to at least two factors, greatly reduced transcript levels and ineffective translation, resulting from mutation of the structural gene encoding the enzyme. In summary, the results indicate that the *Ep* gene regulatory elements can drive high level expression in a tightly coordinated, tissue and developmentally specific manner.

All scientific publications and patent documents are incorporated herein by reference.

The present invention has been described with regard to preferred
embodiments. However, it will be obvious to persons skilled in the art that a number
of variations and modifications can be made without departing from the scope of the
invention as described in the following claims.

[illegible]

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20

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: GIJZEN, Mark
- (ii) TITLE OF INVENTION: SEED COAT SPECIFIC DNA REGULATORY REGION
AND PEROXIDASE
- (iii) NUMBER OF SEQUENCES: 2
- (iv) CORRESPONDENCE ADDRESS:
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 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE: 26-SEP-1997
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/723,414
 - (B) FILING DATE: 30-SEP-1996
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(2) INFORMATION FOR SEQ ID NO: 1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1244 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:1..1056

(ix) FEATURE:

(A) NAME/KEY: sig_peptide

(B) LOCATION:1..77

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATG GGT TCC ATG CGT CTA TTA GTA GTG GCA TTG TTG TGT GCA TTT GCT 48

Met Gly Ser Met Arg Leu Leu Val Val Ala Leu Leu Cys Ala Phe Ala

1 5 10 15

ATG CAT GCA GGT TTT TCA GTC TCT TAT GCT CAG CTT ACT CCT ACG TTC 96

Met His Ala Gly Phe Ser Val Ser Tyr Ala Gln Leu Thr Pro Thr Phe

20 25 30

TAC AGA GAA ACA TGT CCA AAT CTG TTC CCT ATT GTG TTT GGA GTA ATC 144

Tyr Arg Glu Thr Cys Pro Asn Leu Phe Pro Ile Val Phe Gly Val Ile

35 40 45

TTC GAT GCT TCT TTC ACC GAT CCC CGA ATC GGG GCC AGT CTC ATG AGG 192

Phe Asp Ala Ser Phe Thr Asp Pro Arg Ile Gly Ala Ser Leu Met Arg

50

55

60

CTT CAT TTT CAT GAT TGC TTT GTT CAA GGT TGT GAT GGA TCA GTT TTG 240

Leu His Phe His Asp Cys Phe Val Gln Gly Cys Asp Gly Ser Val Leu

65

70

75

80

CTG AAC AAC ACT GAT ACA ATA GAA AGC GAG CAA GAT GCA CTT CCA AAT 288

Leu Asn Asn Thr Asp Thr Ile Glu Ser Glu Gln Asp Ala Leu Pro Asn

85

90

95

ATC AAC TCA ATA AGA GGA TTG GAC GTT GTC AAT GAC ATC AAG ACA GCG 336

Ile Asn Ser Ile Arg Gly Leu Asp Val Val Asn Asp Ile Lys Thr Ala

100

105

110

GTG GAA AAT AGT TGT CCA GAC ACA GTT TCT TGT GCT GAT ATT CTT GCT 384

Val Glu Asn Ser Cys Pro Asp Thr Val Ser Cys Ala Asp Ile Leu Ala

115

120

125

ATT GCA GCT GAA ATA GCT TCT GTT CTG GGA GGA GGT CCA GGA TGG CCA 432

Ile Ala Ala Glu Ile Ala Ser Val Leu Gly Gly Gly Pro Gly Trp Pro

130

135

140

GTT CCA TTA GGA AGA AGG GAC AGC TTA ACA GCA AAC CGA ACC CTT GCA 480

Val Pro Leu Gly Arg Arg Asp Ser Leu Thr Ala Asn Arg Thr Leu Ala

145

150

155

160

AAT CAA AAC CTT CCA GCA CCT TTC TTC AAC CTC ACT CAA CTT AAA GCT 528

Asn Gln Asn Leu Pro Ala Pro Phe Phe Asn Leu Thr Gln Leu Lys Ala

165

170

175

45669 50668

[illegible]

ATTAATCA 1244

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: promoter

(B) LOCATION:1..1532

(ix) FEATURE:

(A) NAME/KEY: sig_peptide

(B) LOCATION:1533..1609

(ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION:1533..1751

(ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION:2383..2574

(ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION:3605..3769

(ix) FEATURE:

(A) NAME/KEY: exon

(B) LOCATION:4033..4516

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION:1752..1782

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION:2575..3604

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION:3770..4032

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:1533..1751

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:2383..2574

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:3605..3769

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:4033..4516

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TAGATAAAAA AATGGGATAT AATTTTCTC AGATGTTGTT TATACTGTTT TTTAATCAG 60

AATTAAAATT CCTCTTTAAT TATCGACATA ATTTTTTTTG GTGAATATTA TCGACATAAT 120

TATTTAATAC AAATTTTAT TGTACATAGA AGTGATACTT CAATTTAAT ATTGGAGAAC 180

AGTACGAAAA CATAAAAAAA CTGTTATTAG AAGAAAAAAA TATATGGAAA AGGTTAGCTA	240
CATATATTAG CTAAATTAGT TGTCTAATT GGCTATATAA ACCCTATTGT ACTCTTTGTA	300
ATCTCACCTT TTTCATTTAA ATACATTTCT ACTTTTAAAG TTCTATAATT TCTCTCAATT	360
TTCTTCGATA AACCATGAAA TTTAACATGG TATATCAGCG ATACCACCCA CTTTGAAAGC	420
CATGTATGGC TAGTATGGGC AGCCAAAATT TGCCCTGGTT CAAGCAAAGC AAGTGTTTAT	480
ATAGATGTGA CTTTGTGTTA GGAATCATG CCAATGGTAC TGATTGTGAA ACTGAGAAAA	540
CTAATTGGA GAATTGAAT TATGATCATT AAATACTCCT CTCCTGACTA CCTTCGTCCC	600
TCAAATTTGT ACCATCATTA TTTCCAAAA ATTTGATTAC AATGCACTAA TTAATGAATG	660
TTCTTACAT TATCATATTA TCATATCTGA CATTTTGTTT TTAATTTTAA TAATAATTAT	720
TTTAAAAAGT CACATGCA AATAATTTT TAATAGTTTA CAGTTAAATT TTTACAGTAA	780
AAATGCATGA AAATTAACT TTATTTTCC AAGTCATCAT TTAGTCAAAT CCCAAAACAA	840
TGATTATTTT TTGCAAATGA ATGTTTATTG AACATTTAAA TGTAGCCTAA TTAATTCTGG	900
TTATGGTGTC AATGTTCCAA AACCTAATGC AAGATCTTAG CAAGTACATA CATAGATCTA	960
ATTTTAACT TATCTTTACG CAAGAGATAT AAAGATTATA CATCTAGTTT TAAACATTAA	1020
CTTTTGTTTT TGTGTTAAAA AACAGTAACA TTTTCTTAAT TTTGTAGAGT GACGTGCTCC	1080
AACCATATTA ACGAAGATTT TAATTGGTAT TCAAGTTCAT GAACTTAGTA AATAAGTTTT	1140

420

GTT CAA GTACGTACTT TTTTTTTTCC TTCCAAAATG CCCTGCATAT TTAACAAGAT 1801

Val Gln

425

TGCTTTGTTC ACCTAGAAAA ATGTGTTTTT TTCAACGATC TTACGTACGT TTGTTTGGTT 1861

TGAAAAATAA ATCAGAAAGA GATCAAGAAA ATAGCTAGAA AGAAAGCAAC GTTTTTTTAA 1921

AAGGTATTTA GTGTGAGAAA AATATTAAAA CTGAAGAGAA AGAAATTAAA TAAGCTTTTC 1981

TTGAATGATA TTTACATGTC TTATTAACCT AAAGTCACCT TTTTCTTTA AGTTGTGCTT 2041

GAAGAAAAAA GATGTCTTTC AGTTTAGTTT TGATTAATGC TAATTATATT TTTAATTAAT 2101

TAATTAATAC TATATATCTA TTTACCATAT TAATTATTAC TATATTTTCAT GATGACAACA 2161

GACAAGTATT CTAAAGAGGT ATCGGTAGAT GATTAATTTT TTTATAAAAA AATCTTTTGC 2221

GTGTATAGAT ATTCTTTTAT AATTGGTGCA GAAACTTGTA ATGCTAATTG CAATTAATCT 2281

TACATTGATT AACTAATAGC TATAATCAAT ATTTAGGTTA GGTATAGGAG ACAAATCAAG 2341

TGATCTGAAC AAATTAAGTT GTTATATTTG CATTGTGACA G GGT TGT GAT GGA 2394

Gly Cys Asp Gly

1

TCA GTT TTG CTG AAC AAC ACT GAT ACA ATA GAA AGC GAG CAA GAT GCA 2442

Ser Val Leu Leu Asn Asn Thr Asp Thr Ile Glu Ser Glu Gln Asp Ala

5

10

15

20

CTT CCA AAT ATC AAC TCA ATA AGA GGA TTG GAC GTT GTC AAT GAC ATC 2490

46660 "006660"

Leu Pro Asn Ile Asn Ser Ile Arg Gly Leu Asp Val Val Asn Asp Ile

25

30

35

AAG ACA GCG GTG GAA AAT AGT TGT CCA GAC ACA GTT TCT TGT GCT GAT

2538

Lys Thr Ala Val Glu Asn Ser Cys Pro Asp Thr Val Ser Cys Ala Asp

40

45

50

ATT CTT GCT ATT GCA GCT GAA ATA GCT TCT GTT CTG GTAATTAATA

2584

Ile Leu Ala Ile Ala Ala Glu Ile Ala Ser Val Leu

55

60

ACTCCTAATT AATCCCAAC CATTAAAAG TTGCATGATT GGATTCAAAA TTCTATGGTA

2644

TTGGGGTTCT GATATAAATT TGTAATTAAA TTGCACTAAA AAAAATTATC ATATACTTTT

2704

AATAAAAAAA ATTTATCTAA TTTAATTTAT TATTAAACT ATTTTAAAA TTCAATCCTA

2764

ACTCTTTTTT AATCGGAGCA TGTAAGCTGG CACCCACCGT ATATCGTTGG AAGATGCTAT

2824

AAAACCATTT AATTAATGGA TGGAATCAGT CAAAACATTT AATTCAAAAT ACTCTTAATT

2884

GTGATTAGTA ATCATGTTTCG GGCAAGTTAC GTTGTGTATA ATTAATTTGA CTTAATCAGA

2944

TAAAAAACA AATGGACGCA AGCCGGTTGG TATAGATATC ACTGGCCTGT AGAATATGTG

3004

GTTTTTCACG TTAAATAAAA AGCTAGCTAC TATATTATAT TTAGTCTTTT TTTTCTTAA

3064

ACCCATTTAA CGTGATTAT TGAAGTGAA ACATGTTTCC ACACACAGGC TTAGAACTC

3124

CTCGCAACTA ACATCTCCAA AATTGACTA TTTATTTATG AAGATAATTC ATCTATGATG

3184

TAGCTAGCAA TAAAAAGTCT CTGATACAGA CATATTTAGA TAAATTAATT TCTCCATAAA 3859

CATTATAAT AAAATTATCA ATTTATGTAC TTAAAAATTA TGGATTGAAG CTCTTTTCAT 3919

CCAACTTTTA CTAAAGTTAA GGTGCATATA ATATAAAATA AACTATCTCT TGTTTCTTAT 3979

AAAAAGATTG AAGATAAGTT AAAGTCTACT TATAAATCAT TAATATATGT ATA GGT 4035

Gly

1

GGT CAT ACG TTT GGA AGA GCT CGG TGC AGT ACA TTC ATA AAC CGA TTA 4083

Gly His Thr Phe Gly Arg Ala Arg Cys Ser Thr Phe Ile Asn Arg Leu

5

10

15

TAC AAC TTC AGC AAC ACT GGA AAC CCT GAT CCA ACT CTG AAC ACA ACA 4131

Tyr Asn Phe Ser Asn Thr Gly Asn Pro Asp Pro Thr Leu Asn Thr Thr

20

25

30

TAC TTA GAA GTA TTG CGT GCA AGA TGC CCC CAG AAT GCA ACT GGG GAT 4179

Tyr Leu Glu Val Leu Arg Ala Arg Cys Pro Gln Asn Ala Thr Gly Asp

35

40

45

AAC CTC ACC AAT TTG GAC CTG AGC ACA CCT GAT CAA TTT GAC AAC AGA 4227

Asn Leu Thr Asn Leu Asp Leu Ser Thr Pro Asp Gln Phe Asp Asn Arg

50

55

60

65

TAC TAC TCC AAT CTT CTG CAG CTC AAT GGC TTA CTT CAG AGT GAC CAA 4275

Tyr Tyr Ser Asn Leu Leu Gln Leu Asn Gly Leu Leu Gln Ser Asp Gln

70

75

80

GAA CTT TTC TCC ACT CCT GGT GCT GAT ACC ATT CCC ATT GTC AAT AGC 4323

Glu Leu Phe Ser Thr Pro Gly Ala Asp Thr Ile Pro Ile Val Asn Ser

85

90

95

446626590596680

TTC AGC AGT AAC CAG AAT ACT TTC TTT TCC AAC TTT AGA GTT TCA ATG 4371
Phe Ser Ser Asn Gln Asn Thr Phe Phe Ser Asn Phe Arg Val Ser Met
100 105 110

ATA AAA ATG GGT AAT ATT GGA GTG CTG ACT GGG GAT GAA GGA GAA ATT 4419
Ile Lys Met Gly Asn Ile Gly Val Leu Thr Gly Asp Glu Gly Glu Ile
115 120 125

CGC TTG CAA TGT AAT TTT GTG AAT GGA GAC TCG TTT GGA TTA GCT AGT 4467
Arg Leu Gln Cys Asn Phe Val Asn Gly Asp Ser Phe Gly Leu Ala Ser
130 135 140 145

GTG GCG TCC AAA GAT GCT AAA CAA AAG CTT GTT GCT CAA TCT AAA TAA 4515
Val Ala Ser Lys Asp Ala Lys Gln Lys Leu Val Ala Gln Ser Lys *
150 155 160

ACCAATAATT AATGGGGATG TGCATGCTAG CTAGCATGTA AAGGCAAATT AGGTTGTAAA 4575

CCTCTTTGCT AGCTATATTG AAATAAACCA AAGGAGTAGT GTGCATGTCA ATTCGATTTT 4635

GCCATGTACC TCTTGAATA TTATGTAATA ATTATTTGAA TCTCTTTAAG GTACTTAATT 4695

AATCA 4700

456350"50666690

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OF PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. An isolated DNA molecule comprising the nucleotide sequence of SEQ ID NO:1.
2. An isolated DNA molecule comprising at least 24 contiguous nucleotides selected from nucleotides 1-1532 of SEQ ID NO:2
3. The isolated DNA molecule comprising a nucleotide sequence substantially homologous to nucleotides 1533-4700 of SEQ ID NO:2.
4. The isolated DNA molecule of claim 3 comprising a nucleotide sequence substantially homologous to that of nucleotides 1-4700 of SEQ ID NO:2.
5. The isolated DNA molecule of claim 3 comprising nucleotides 1533-4700 of SEQ ID NO:2.
6. The isolated DNA molecule of claim 4 comprising the nucleotide sequence of SEQ ID NO:2.
7. The isolated DNA molecule of claim 2 comprising a nucleotide sequence substantially homologous to that of 1-1532 of SEQ ID NO:2.
8. The isolated DNA molecule of claim 7, comprising the nucleotide sequence of nucleotides 1-1532 of SEQ ID NO:2.
9. An isolated DNA molecule of claim 3 comprising at least 32 contiguous nucleotides selected from nucleotides 412-1041 of SEQ ID NO:2.

10. An isolated DNA molecule of claim 9 comprising the nucleotide sequence of 412-1041 of SEQ ID NO:2.
11. An isolated DNA molecule of claim 3 comprising at least 23 contiguous nucleotides selected from nucleotides 1234-2263 of SEQ ID NO:2.
12. An isolated DNA molecule of claim 11 comprising the nucleotide sequence of 1234-2263 of SEQ ID NO:2.
13. An isolated DNA molecule of claim 3 comprising at least 22 contiguous nucleotides selected from nucleotides 2430-2691 of SEQ ID NO:2.
14. An isolated DNA molecule of claim 13 comprising the nucleotide sequence of 2430-2691 of SEQ ID NO:2.
15. A vector which comprises the DNA molecule of claim 1.
16. A vector which comprises the DNA molecule of claim 2.
17. A vector which comprises the DNA molecule of claim 3.
18. The vector of claim 16 which comprises a heterologous gene of interest under control of the DNA molecule.
19. A host cell capable of expressing the DNA molecule within the vector of claim 15.
20. A host cell capable of expressing the DNA molecule within the vector of claim 16.

21. A host cell capable of expressing the DNA molecule within the vector of claim 17.
22. A host cell capable of expressing the DNA molecule within the vector of claim 18.
23. A transgenic plant comprising the vector of claim 15.
24. A transgenic plant comprising the vector of claim 16.
25. A transgenic plant comprising the vector of claim 17.
26. A transgenic plant comprising the vector of claim 18.
27. A method for the production of soybean seed coat peroxidase in a host cell comprising:
 - i) transforming the host cell with a vector comprising an isolated DNA molecule selected from the group consisting of SEQ ID NO:1, and SEQ ID NO:2; and
 - ii) culturing the host cell under conditions to allow expression of the soybean seed coat peroxidase.
28. A process for producing a heterologous gene of interest comprising propagating a transformed plant with the vector of claim 16.
29. The process of claim 28 wherein the heterologous gene of interest is produced within seed coat cells.

ABSTRACT OF THE DISCLOSURE

A novel seed coat specific peroxidase genomic sequence is characterized and presented. Adjacent DNA regulatory regions have also been characterized. The seed coat peroxidase is translated as a 352 amino acid precursor protein of 38 kDa comprising a 26 amino acid signal sequence which when cleaved results in a 35 kDa protein. Plants containing a dominant *Ep* allele accumulate large amounts of peroxidase in the hourglass cells of the subepidermis. Homozygous recessive *epep* genotypes do not accumulate peroxidase in the hourglass cells and are much reduced in total seed coat peroxidase activity. Probes derived from the cDNA, or genomic DNA can be used to detect polymorphisms that distinguished *EpEp* and *epep* genotypes. Cosegregation of the polymorphisms in an F₂ population from a cross of *EpEp* and *epep* plants shows that the *Ep* locus encodes the seed coat peroxidase protein. Comparison of *Ep* and *ep* alleles indicates that the recessive gene lacks 87 bp of sequence encompassing the translation start codon. The heterologous expression, as well as vectors and hosts to be used for the expression of the seed coat peroxidase, are also disclosed. The seed-specific DNA regulatory region may be used to control expression of genes of interest such as i) genes encoding herbicide resistance, or ii) biological control of insects or pathogens (e.g. *B. thuringiensis*), or iii) viral coat proteins to protect against viral infections, or iv) proteins of commercial interest (e.g. pharmaceutical), and v) proteins that alter the nutritive value, taste, or processing of seeds.

1970-1971		1971-1972		1972-1973		1973-1974		1974-1975		1975-1976		1976-1977		1977-1978		1978-1979		1979-1980		1980-1981		1981-1982		1982-1983		1983-1984		1984-1985		1985-1986		1986-1987		1987-1988		1988-1989		1989-1990		1990-1991		1991-1992		1992-1993		1993-1994		1994-1995		1995-1996		1996-1997		1997-1998		1998-1999		1999-2000		2000-2001		2001-2002		2002-2003		2003-2004		2004-2005		2005-2006		2006-2007		2007-2008		2008-2009		2009-2010		2010-2011		2011-2012		2012-2013		2013-2014		2014-2015		2015-2016		2016-2017		2017-2018		2018-2019		2019-2020		2020-2021		2021-2022		2022-2023		2023-2024		2024-2025		2025-2026		2026-2027		2027-2028		2028-2029		2029-2030		2030-2031		2031-2032		2032-2033		2033-2034		2034-2035		2035-2036		2036-2037		2037-2038		2038-2039		2039-2040		2040-2041		2041-2042		2042-2043		2043-2044		2044-2045		2045-2046		2046-2047		2047-2048		2048-2049		2049-2050		2050-2051		2051-2052		2052-2053		2053-2054		2054-2055		2055-2056		2056-2057		2057-2058		2058-2059		2059-2060		2060-2061		2061-2062		2062-2063		2063-2064		2064-2065		2065-2066		2066-2067		2067-2068		2068-2069		2069-2070		2070-2071		2071-2072		2072-2073		2073-2074		2074-2075		2075-2076		2076-2077		2077-2078		2078-2079		2079-2080		2080-2081		2081-2082		2082-2083		2083-2084		2084-2085		2085-2086		2086-2087		2087-2088		2088-2089		2089-2090		2090-2091		2091-2092		2092-2093		2093-2094		2094-2095		2095-2096		2096-2097		2097-2098		2098-2099		2099-2100		2100-2101		2101-2102		2102-2103		2103-2104		2104-2105		2105-2106		2106-2107		2107-2108		2108-2109		2109-2110		2110-2111		2111-2112		2112-2113		2113-2114		2114-2115		2115-2116		2116-2117		2117-2118		2118-2119		2119-2120		2120-2121		2121-2122		2122-2123		2123-2124		2124-2125		2125-2126		2126-2127		2127-2128		2128-2129		2129-2130		2130-2131		2131-2132		2132-2133		2133-2134		2134-2135		2135-2136		2136-2137		2137-2138		2138-2139		2139-2140		2140-2141		2141-2142		2142-2143		2143-2144		2144-2145		2145-2146		2146-2147		2147-2148		2148-2149		2149-2150		2150-2151		2151-2152		2152-2153		2153-2154		2154-2155		2155-2156		2156-2157		2157-2158		2158-2159		2159-2160		2160-2161		2161-2162		2162-2163		2163-2164		2164-2165		2165-2166		2166-2167		2167-2168		2168-2169		2169-2170		2170-2171		2171-2172		2172-2173		2173-2174		2174-2175		2175-2176		2176-2177		2177-2178		2178-2179		2179-2180		2180-2181		2181-2182		2182-2183		2183-2184		2184-2185		2185-2186		2186-2187		2187-2188		2188-2189		2189-2190		2190-2191		2191-2192		2192-2193		2193-2194		2194-2195		2195-2196		2196-2197	
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1970-71		1971-72		1972-73		1973-74		1974-75		1975-76		1976-77		1977-78		1978-79		1979-80		1980-81		1981-82		1982-83		1983-84		1984-85		1985-86		1986-87		1987-88		1988-89		1989-90		1990-91		1991-92		1992-93		1993-94		1994-95		1995-96		1996-97		1997-98		1998-99		1999-00		2000-01		2001-02		2002-03		2003-04		2004-05		2005-06		2006-07		2007-08		2008-09		2009-10		2010-11		2011-12		2012-13		2013-14		2014-15		2015-16		2016-17		2017-18		2018-19		2019-20		2020-21		2021-22		2022-23		2023-24		2024-25		2025-26		2026-27		2027-28		2028-29		2029-30		2030-31		2031-32		2032-33		2033-34		2034-35		2035-36		2036-37		2037-38		2038-39		2039-40		2040-41		2041-42		2042-43		2043-44		2044-45		2045-46		2046-47		2047-48		2048-49		2049-50		2050-51		2051-52		2052-53		2053-54		2054-55		2055-56		2056-57		2057-58		2058-59		2059-60		2060-61		2061-62		2062-63		2063-64		2064-65		2065-66		2066-67		2067-68		2068-69		2069-70		2070-71		2071-72		2072-73		2073-74		2074-75		2075-76		2076-77		2077-78		2078-79		2079-80		2080-81		2081-82		2082-83		2083-84		2084-85		2085-86		2086-87		2087-88		2088-89		2089-90		2090-91		2091-92		2092-93		2093-94		2094-95		2095-96		2096-97		2097-98		2098-99		2099-00		2100-01		2101-02		2102-03		2103-04		2104-05		2105-06		2106-07		2107-08		2108-09		2109-10		2110-11		2111-12		2112-13		2113-14		2114-15		2115-16		2116-17		2117-18		2118-19		2119-20		2120-21		2121-22		2122-23		2123-24		2124-25		2125-26		2126-27		2127-28		2128-29		2129-30		2130-31		2131-32		2132-33		2133-34		2134-35		2135-36		2136-37		2137-38		2138-39		2139-40		2140-41		2141-42		2142-43		2143-44		2144-45		2145-46		2146-47		2147-48		2148-49		2149-50		2150-51		2151-52		2152-53		2153-54		2154-55		2155-56		2156-57		2157-58		2158-59		2159-60		2160-61		2161-62		2162-63		2163-64		2164-65		2165-66		2166-67		2167-68		2168-69		2169-70		2170-71		2171-72		2172-73		2173-74		2174-75		2175-76		2176-77		2177-78		2178-79		2179-80		2180-81		2181-82		2182-83		2183-84		2184-85		2185-86		2186-87		2187-88		2188-89		2189-90		2190-91		2191-92		2192-93		2193-94		2194-95		2195-96		2196-97		2197-98		2198-99		2199-00		2200-01		2201-02		2202-03		2203-04		2204-05		2205-06		2206-07		2207-08		2208-09		2209-10		2210-11		2211-12		2212-13		2213-14		2214-15		2215-16		2216-17		2217-18		2218-19		2219-20		2220-21		2221-22		2222-23		2223-24		2224-25	
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FIGURE 2

	10	20	30	40	50	60
1	GCATCATATCATAAACAATACGTACGTGATATTATCTAGTGTCTCTCAGTTTACTTTTATG					
61	AGAAATTATTTTTCTTTAAAAAAGTTAATTAATAAAAAACATTTGCGATACCGTGAGTTA					
121	CAAGAAATCCGCCGAATTCATCTCTATAAATAAAAGGATCTATATGAGAGGTAAAATCAT					
181	ATTAAC TCAAAATGGGTTCCATGCGTCTATTAGTAGTGGCATTGTTGTGTGCATTTGCTA					
241	TGCATGCAGGTTTTTCAGTCTCTTATGCTCAGCTTACTCCTACGTTCTACAGAGAAACAT					
301	GTCCAAATCTGTTCCCTATTGTGTTTGGAGTAATCTTCGATGCTTCTTTACACCGATCCCC					
361	GAATCGGGGCCAGTCTCATGAGGCTTCATTTTTCATGATTGCTTTGTTCAAGTACGTACTT					
421	TTTTTTTTCTTCCAAAATGCCCTGCATATTTAACAAGATTGCTTTGTTTACCTAGAAAA					
481	ATGTGTTTTTTTTCAACGATCTTACGTACGTTTGTGTTTGGTTTGAAAAATAAATCAGAAAGA					
541	GATCAAGAAAATAGCTAGAAAGAAAGCAACGTTTTTTTTTAAAAGGTATTTAGTGTGAGAAA					
601	AATATTAAAACTGAAGAGAAAGAAATTAAATAAGCTTTTCTTGAATGATATTTACATGTC					
661	TTATTAAC TTAAGTCACCTTTTTCTTTAAGTTGTGCTTGAAGAAAAAAGATGTCTTTC					
721	AGTTTAGTTTTTGATTAATGCTAATTATATTTTAAATTAATTAATACTATATATCTA					
781	TTTACCATATTAATTATTACTATATTTTCATGATGACAACAGACAAGTATTCTAAAGAGGT					
841	ATCGGTAGATGATTAATTTTTTTTATAAAAAAATCTTTTGCGTGTATAGATATTCTTTTAT					
901	AATTGGTGCAGAACTTGTAATGCTAATTGCAATTAATCTTACATTGATTAATAATAGC					
961	TATAATCAATATTTAGGTTAGGTATAGGAGACAAATCAAGTGATCTGAACAAATTAAGTT					
1021	GTTATATTTGCATTGTGACAGGGTTGTGATGGATCAGTTTTGCTGAACAACACTGATACA					
1081	ATAGAAAGCGAGCAAGATGCACTTCCAAATATCAACTCAATAAGAGGATTGGACGTTGTC					
1141	AATGACATCAAGACAGCGGTGGAAAATAGTTGTCCAGACACAGTTTCTTGTGCTGATATT					
1201	CTTGCTATTGCAGCTGAAATAGCTTCTGTTCTGGTAATTAATAACTCCTAATTAATTCCC					
1261	AACCATTAAAAAGTTGCATGATTGGATTCAAAATTCTATGGTATTGGGGTTCTGATATAA					
1321	ATTTGTAATTAAATTGCACTAAAAAAATTATCATATACTTTTAATAAAAAAAATTTATC					
1381	TAATTTAATTTATTATTAAACTATTTTTTAAATTCATCCTAACTCTTTTTTAATCGGA					
1441	GCATGTAAGCTGGCACCCACCGTATATCGTTGGAAGATGCTATAAAACCATTTAATTAAT					
1501	GGATGGAATCAGTCAAAACATTTAATTCAAAATACTCTTAATTGTGATTAGTAATCATGT					
1561	TCGGGCAAGTTACGTTGTGTATAATTAATTTGACTTAATCAGATAAAAAAACAAATGGAC					
1621	GCAAGCCGGTTGGTATAGATATCACTGGCCTGTAGAATATGTGGTTTTTTCACGTTTAAAT					
1681	AAAAGCTAGCTACTATATTATTTAGTCTTTTTTTTTTCTTAAACCCATTTAACGTGATT					
1741	TATTGACTGTGAAACATGTTTCCACACACAGGCTTAGAACTCCTCGCAACTAACATCTC					
1801	CAAAATTTGACTATTTATTTATGAAGATAATTCATCTATGATGTTCAACTCTATTATATA					
1861	TATGTATCATCGCAGTATTAAGAATTATAATAGTCAAATATAGAAGTATATCGGGTAAAT					
1921	GTAGTTGCATGTGCGACCTGTTTCGTGTAAAATGCTTATTCTATATAGCTTTTTTTTATTG					
1981	GAAAATAACGATGAACTAAAAACGAAAGGGTATCATATAGTTTGACTTTTATGTTAGAGA					
2041	GAGACATCTTAATTTGGTCATATGTTAAATAATTAATTACAATGCATACACAAATATTTA					
2101	TGCCATATCTAAAAAATGATAAAATATCATAGGTATACTCAACTATATGATATCCCCATA					
2161	ACAGAAATTGTACTTTTCTTCAGGCAATGAACTTAACATTTCTGTTTGCTAAAAACAAAC					
2221	ATCCACTTAAAGTGGTTCAACATATTTATGTAATAATTTACAGGGAGGAGGTCCAGGATG					
2281	GCCAGTTCCATTAGGAAGAAGGGACAGCTTAACAGCAAACCGAACCCTTGCAAATCAAAA					
2341	CCTTCCAGCACCTTTCTTCAACCTCACTCAACTTAAAGCTTCCTTTGCTGTTCAAGGTCT					
2401	CAACACCCTTGATTTAGTTTACACTCTCAGGTATACATAATCAATTTTTTTATTTGCTATTA					
2461	GCTAGCAATAAAAAGTCTCTGATACAGACATATTTAGATAAATTAATTTCTCCATAAACA					
2521	TTTATAATAAAATTATCAATTTATGTACTTAAAAATTATGGATTGAAGCTCTTTTCATCC					
2581	AACTTTTACTAAAGTTAAGGTGCATATAATATAAAATAAACTATCTCTTGTTCCTTATAA					
2641	AAAGATTGAAGATAAGTTAAAGTCTACTTATAAATCATTAATATATGTATAGGTGGTCAT					
2701	ACGTTTGGAAGAGCTCGGTGCAGTACATTCATAAACCGATTATACAACTTCAGCAACACT					
2761	GGAAACCCTGATCCAACCTCTGAACACAACATACTTAGAAGTATTGCGTGCAAGATGCCCC					
2821	CAGAATGCAACTGGGGATAACCTCACCAATTTGGACCTGAGCACACCTGATCAATTTGAC					
2881	AACAGATACTACTCCAATCTTCTGCAGCTCAATGGCTTACTTCAGAGTGACCAAGAACTT					
2941	TTCTCCACTCCTGGTGCTGATACCATTCCCATTGTCAATAGCTTCAGCAGTAACCAGAAT					
3001	ACTTCTTTTCCAACCTTTAGAGTTTCAATGATAAAAATGGGTAATATTGGAGTGCTGACT					

3061 GGGGATGAAGGAGAAATTCGCTTGCAATGTAATTTTGTGAATGGAGACTCGTTTGGATTA
3121 GCTAGTGTGGCGTCCAAAGATGCTAAACAAAAGCTTGTTGCTCAATCTAAATAAACCAAT
3181 AATTAATGGGGATGTGCATGCTAGCTAGCATGTAAAGGCAAATTAGGTTGTAAACCTCTT
3241 TGCTAGCTATATTGAAATAAACCAAAGGAGTAGTGTGCATGTCAATTCGATTTTGCCATG
3301 TACCTCTTGGAATATTATGTAATAATTATTTGAATCTCTTTAAGGTACTTAATTAATCA

FIGURE 3A

L78163	-----ATGGGTTCATGCGT-CTATTAGTAGTGGCATTGTTG	36
U41657	-----	0
X90693	G----GCAAA-CAATGAACTCCCTTCGTGCTGTAGCAATAG-CTTTGTGC	44
X90694	GCTCTTCAAAACAATGAACTCC-----TTAGCAACTT-CTATGTGG	40
L36156	-----CTCC-----TTAGCAACTT-CTATGTGG	22
X90692	-----AATGCTTGGT-----CTAAGTGCAACAGCTTTTTTGCTGTATGG	38
L78163	TGT-----GCATTT-GCTATGCATGCAGGTTTTTTCAGT---CTCTTATGC	77
U41657	-----	0
X90693	TGTATTGTG-----GTTGTGCTTGGAGGGTTACCCTTCTCTTCAAATGC	88
X90694	TGTGTTGTGCTTTTAGTTGTGCTTGGAGGACTACCCTTTTCCTCAGATGC	90
L36156	TGTGTTGTGCTTTTAGTTGTGCTTGGAGGACTACCCTTTTCCTCAGATGC	72
X90692	TGT-TTGTGCTAAT-----TGGAGGAGTACCCTTTT---CAAATGC	75
L78163	TCAGCTTACTCCTACGTTCTACAGAGAAACATGTCCAAATCTGTTCCCTA	127
U41657	-----	0
X90693	GCAACTTGATCCATCCTTTTACAGGAACACTTGTCCAAATGTTAGTTCCA	138
X90694	ACAACCTTAGTCCCACCTTTTACAGCAAAACGTGTCCAACCTGTTAGTTCCA	140
L36156	ACAACCTTAGTCCCACCTTTTACAGCAAAACGTGTCCAACCTGTTAGTTCCA	122
X90692	ACAACCTAGATCCTTCATTTTACAACAGTACATGTTCTAATCTTGATTCAA	125
L78163	TTGTGTTTGGAGTAATCTTCGATGCTTCTTTCACCGATCCCCGAATCGGG	177
U41657	-----	0
X90693	TTGTTTCGTGAAGTCATAAGGAGTGTTTCTAAGAAAGATCCTCGTATGCTT	188
X90694	TTGTTAGCAATGTCTTAACAAACGTTTCTAAGACAGATCCTCGCATGCTT	190
L36156	TTGTTAGCAATGTCTTAACAAACGTTTCTAAGACAGATCCTCGCATGCTT	172
X90692	TCGTACGTGGTGTGCTCACAAATGTTTCACAATCTGATCCCAGAATGCTT	175
L78163	GCCAGTCTCATGAGGCTTCATTTTCATGATTGCTTTGTTCAAGGTGTGA	227
U41657	-----TTTCATGATTGCTTTGTTCAAGGTGTGA	29
X90693	GCTAGTCTTGTGAGGCTTCACTTTCATGACTGTTTTGTTCAAGGTGTGA	238
X90694	GCTAGTCTCGTCAGGCTTCACTTTCATGACTGTTTTGTTCTGGGATGTGA	240
L36156	GCTAGTCTCGTCAGGCTTCACTTTCATGACTGTTTTGTTCTGGGATGTGA	222
X90692	GGTAGTCTCATCAGGCTACATTTTCATGACTGTTTTGTTCAAGGTGCGA	225
	***** ** *****..**.* *	
L78163	TGGATCAGTTTTGCTGAACAACACTGATACAATAGAAAGCGAGCAAGATG	277
U41657	TGGATCAGTTTTACTGAACAACACTGATACAATAGAAAGCGAGCAAGATG	79
X90693	TGCATCAGTTTTACTAAACAAACACTGATACCGTTGTGAGTGAACAAGATG	288
X90694	TGCCTCAGTTTTGCTGAACAATACTGCTACAATCGTAAGCGAACAACAAG	290
L36156	TGCCTCAGTTTTGCTGAACAATACTGCTACAATCGTAAGCGAACAACAAG	272
X90692	TGCCTCGATTTTGTGCTGAACGATACGGCTACAATAGTGAGCGAGCAAAGTG	275
	** **..*****..**.* **.* *** .* *..** **.* ** ..*	
L78163	CACTTCCAAATATCAACTCAATAAGAGGATTGGACGTTGTCAATGACATC	327
U41657	CACTTCCAAATATCAACTCAATAAGAGGATTGGACGTTGTCAATGACATC	129
X90693	CTTTTCCAAACAGAACTCATTAAGAGGTTTGGATGTTGTGAATCAAATC	338
X90694	CTTTTCCAAATAACAACCTCTCTAAGAGGTTTGGATGTTGTGAATCAGATC	340
L36156	CTTTTCCAAATAACAACCTCTCTAAGGGGTTTGGATGTTGTGAATCAGATC	322
X90692	CACCACCAATAACAACCTCCATAAGAGGTTTGGATGTGATAAACCAGATC	325
	*. ***** *. ***** ***** **.* ** * ***	
L78163	AAGACAGCGGTGGAAAATAGTTGTCCAGACACAGTTTCTTGTGCTGATAT	377

X90693	GATTGTACAACTTCAGCGGTACGGGAAGTCCCGATCCAACCTCTTAACACA	683
X90694	GATTGTACAACTTCAGCAGTACTGGAAGTCCCGATCCAACCTCTTAACACA	685
L36156	GATTGTACAACTTCAGCAGTACTGGAAGTCCCGATCCAACCTCTTAACACA	664
X90692	GATTATACAAATTTTCAGCAACACTGGAAACCCCGATTCAACCTCTTAACACG	670
	****.***** *****. . **.* ** * ** *..*****.	
L78163	ACATACTTAGAAGTATTGCGTGCAAGATGCCCCCAGAATGCAACTGGGGA	722
U41657	ACATACTTAGAAGTATTGCGTGCAAGATGCCCCCAGAATGCAACTGGGGA	518
X90693	ACTTACTTACAACAATTGCGCACAATATGTCCCAATGGTGGACCTGGCAC	733
X90694	ACTTACTTACAACAACACTGCGCACAATATGTCCCAATGGTGGACCTGGCAC	735
L36156	ACTTACTTACAACAACACTGCGCACAATATGTCCCAATGGTGGACCTGGCAC	714
X90692	ACCTATTTACAACATTGCAAGCAATATGTCCCAATGGTGGACCTGGTAC	720
	** ** *** ** * ***. .***.*** *** *..** * **** .	
L78163	TAACCTCACCAATTTGGACCTGAGCACACCTGATCAATTTGACAACAGAT	772
U41657	TAACCTCACCAATTTGGACCTGAGCACACCTGATCAATTTGACAACAGAT	568
X90693	GAACCTTACCAATTTTCGATCCAACGACTCCTGATAAATTTGACAAGAACT	783
X90694	AAACCTTACCAATTTTCGATCCAACGACTCCTGATAAATTTGACAAGAACT	785
L36156	AAACCTTACCAATTTTCGATCCAACGACTCCTGATAAATTTGACAAGAACT	764
X90692	AAACCTAACCGATTTGGACCCAACCACACCAGATACATTTGACTCCAAC	770
	.***** ***.***** ** * . * **.***.*** *****. * . *	
L78163	ACTACTCCAATCTTCTGCAGCTCAATGGCTTACTTCAGAGTGACCAAGAA	822
U41657	ACTACTCCAATCTTCTGCAGCTCAATGGCTTACTTCAGAGTGACCAAGAA	618
X90693	ATTACTCTAATCTTCAAGTGAAAAAAGGTTTGCTTCAAAGTGATCAAGAG	833
X90694	ATTACTCCAATCTTCAAGTGAAAAAAGGGTTTGCTTCAAAGTGATCAAGAG	835
L36156	ATTACTCCAATCTTCAAGTGAAAAAAGGGTTTGCTTCAAAGTGATCAAGAG	814
X90692	ACTACTCCAATCTCCAAGTTGGAAAGGGCTTGTTTCAGAGTGACCAAGAG	820
	* ***** ***** *.. . . **.* ** * **.****** *****.	
L78163	CTTTTCTCCACTCCTGGTGCTGATACCATTTCCCATTTGTCAATAGCTTCAG	872
U41657	CGTTTCTCCACTCCTGGTGCTGATACCATTTCC-ATTGTCAATAGCTTCAG	667
X90693	TTGTTCTCAACATCTGGTTCAGATACCATTTAGCATTGTCAACAAATTCGC	883
X90694	TTGTTCTCAACTTCTGGTGCTGATACCATTTAGCATTGTCAACAAATTCAG	885
L36156	TTGTTCTCAACTTCTGGTGCTGATACCATTTAGCATTGTGACAAATTCAG	864
X90692	CTTTTTTCCAGAAATGGTTCTGACACTATTTCTATTGTCAATAGTTTCGC	870
	..** ** * . *****.* ** ** * *****.* * . ***.	
L78163	CAGTAACCAGAATACTTTCTTTTCCAACTTTAGAGTTTCAATGATAAAAA	922
U41657	CG--AACCAGAATACTTTCTTTTCCAACTTTAGAGTTTCAATGATAAAAA	715
X90693	AACCGATCAAAAAGCTTTTTTTTGAGAGCTTTAGGGCTGCTATGATCAAAA	933
X90694	CACCGATCAAAAATGCTTTCTTTGAGAGCTTTAAGGCTGCAATGATTAAAA	935
L36156	CACCGATCAAAAATGCTTTCTTTGAGAGCTTTAAGGCTGCAATGATTAAAA	914
X90692	CAATAATCAAACCTCTCTTCTTTGAAAATTTTGTAGCTCAATGATAAAAA	920
	. . * **.* . ** ***. * . ***...* .*.***** ****	
L78163	TGGGTAATATTGGAGTGCTGACTGGGGATGAAGGAGAAATTCGCTTGCAA	972
U41657	TGGGTAATATTGGAGTGCTGACTGGGGATGAAGGAGAAATTCGCTTGCAA	765
X90693	TGGGAAATATTGGTGTGTTAACC GGGAACCAAGGAGAGATTAGAAAACAA	983
X90694	TGGGCAATATTGGTGTGCTAACAGGGACAAAAGGAGAGATTAGAAAACAA	985
L36156	TGGGCAATATTGGTGTGCTAACAGGGACAAAAGGAGAGATTAGAAAACAA	964
X90692	TGGGTAATATTGGAGTTTTA ACTGGATCTCAAGGTGAAATTAGAACACAG	970

FIGURE 3B

L78163	MGSMRLLVALLCAFAMHAGFSVSY---AQLTPTFYRETCPNLFPIVFGV	47
U41657	-----	0
X90693	MNSLRAVAIALCCIV--VVLGGLPFSSNAQLDPSFYRNTCPNVSSIVREV	48
X90694	MNSL---ATSMWCVVLLVVLGGLPFSSDAQLSPTFYSKTCPTVSSIVSNV	47
L36156	M-----WCVVLLVVLGGLPFSSDAQLSPTFYSKTCPTVSSIVSNV	40
X90692	MLGLSATA---FCCMVFLIGGVFVS-NAQLDPSFYNSTCSNLDIVRGV	46
L78163	IFDASFTDPRIGASLMRLHFHDCFVQGCDSVLLNNTDTIESEQDALPNI	97
U41657	-----FHDCEFVQGCDSVLLNNTDTIESEQDALPNI	31
X90693	IRSVSKKDPRMLASLVRLHFHDCFVQGCDAVLLNKTDTVVSEQDAFPNR	98
X90694	LTNVSKTDPRMLASLVRLHFHDCFVLGCDASVLLNNTATIVSEQQAFPNN	97
L36156	LTNVSKTDPRMLASLVRLHFHDCFVLGCDASVLLNNTATIVSEQQAFPNN	90
X90692	LTNVSQSDPRMLGSLIRLHFHDCFVQGCDAVLLNNTATIVSEQSAPPNN	96
	***** ***.****.*** ** *	
L78163	NSIRGLDVVNDIKTAVENSCPDTVSCADILAIAAEIASVLGGPGWPVPL	147
U41657	NSIRGLDVVNDIKTAVENSCPDTVSCADILAIAAEIASVAGRRSGWPVPL	81
X90693	NSLRGLDVVNQIKTAVEKACPNTVSCADILALSAELSTLADGPDWKVPL	148
X90694	NSLRGLDVVNQIKLAVEVPCPNTVSCADILALAAQASSVLAQGPPSWTVPL	147
L36156	NSLRGLDVVNQIKTAVESACPNTVSCADILALA-QASSVLAQGPPSWTVPL	139
X90692	NSIRGLDVINQIKTAVENACPNTVSCADILALSAEISSDLANGPTWQVPL	146
	.**.*** ***.*****.*** ** *	
L78163	GRRDSL TANRTLANQNLPAPFFNL TQLKASFAVQGLNTLDLVTLSGGHTF	197
U41657	GRRDSL TANRTLANQNLPAPFFNL TQLKASFAVQGLNTLDLVTLSGGHTS	131
X90693	GRRDGLTANQLLANQNLPAPFNTTDLKAAFAAQGLD TDLVALSGAHTF	198
X90694	GRRDGLTANRTLANQNLPAPFNSLDQLKAAFTAQGLNTTDLVALSGAHTF	197
L36156	GRRDGLTANRTLANQNLPAPFNSLDHLKLHLTAQGLITPVLVALSGAHTF	189
X90692	GRRDSL TANNSLAAQNLPAPTFNLTRLKSNFDNQNLS TDLVALSGGHTI	196
	****.*****.***.*****.*** ** *	
L78163	GRARCSTFINRLYNFSNTGNPDPTLNTTYLEVLRARCPQONATGDNLTNLD	247
U41657	GRARCSTFINRLYNFSNTGLIH--LDTTYLEVLRARCPQONATGDNLTNLD	179
X90693	GRAHCSLFVSRLYNFSGTGSPDPTLNTTYLQQLRTICPNGGPGTNLTNFD	248
X90694	GRAHCAQFVSRLYNFSSTGSPDPTLNTTYLQQLRTICPNGGPGTNLTNFD	247
L36156	GRAHCAQFVSRLYNFSSTGSPDPTLNTTYLQQLRTICPNGGPGTNLTNFD	239
X90692	GRGQCRFFVDRLYNFSNTGNPDSTLNTTYLQTLQAICPNGGPGTNLTDL	246
	. ***.*****.*** ***.*****.*** ** *	
L78163	LSTPDQFDNRYYSNLLQLNGLLQSDQELFSTPGADTIPIVNSFSSNQNTF	297
U41657	LSTPDQFDNRYYSNLLQLNGLLQSDQERFSTPGADTIPLSIA-SANQNTF	228
X90693	PTTPDKFDKNYYSNLQVKKGLLQSDQELFSTSGSDTISIVNKFATDQKAF	298
X90694	PTTPDKFDKNYYSNLQVKKGLLQSDQELFSTSGADTISIVNKFSTDQNAF	297
L36156	PTTPDKFDKNYYSNLQVKKGLLQSDQELFSTSGADTISIVDKFSTDQNAF	289
X90692	PTTPDTFDSNYYSNLQVGKGLFQSDQELFSRNGSDTISIVNSFANNQTLF	296
	*** ***.*****.***.*****.*** ** *	
L78163	FSNFRVSMIKMGNIGVLTGDEGEIRLQCNFVN----GDSFGLASVAS-K	341
U41657	FSNFRVSMIKMGNIGVLTGDEGEIRLQCNFVN----GDSFGLASVAS-K	272
X90693	FESFRAAMIKMGNIGVLTGNQGEIRKQCNFVN---SKSAELGLINVAS-A	344
X90694	FESFKAAMIKMGNIGVLTGTKGEIRKQCNFVNFSNSAELDLATIASIV	347
L36156	FESFKAAMIKMGNIGVLTGTKGEIRKQCNFVN---SNSAELDLATIASIV	336

FIGURE 4

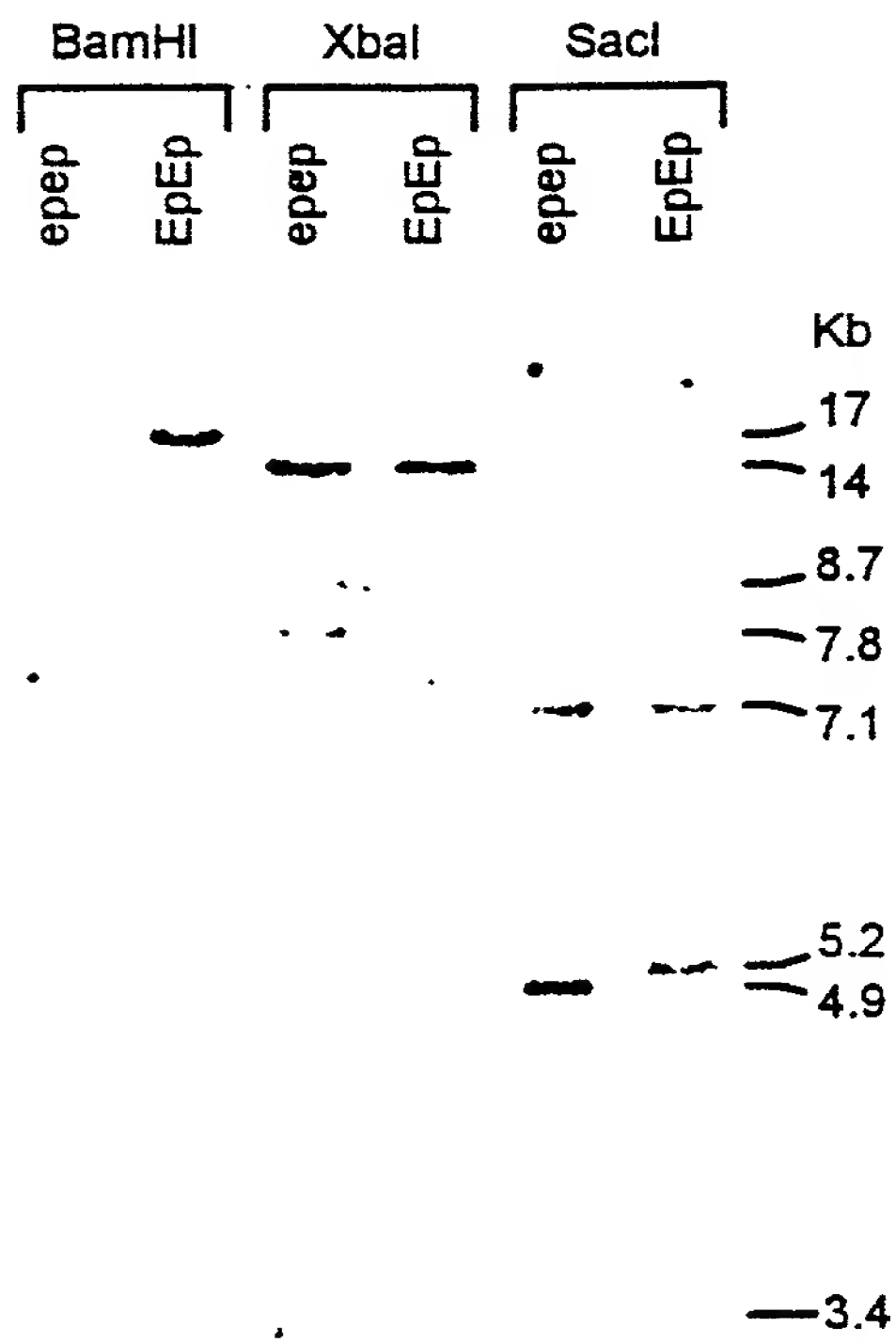
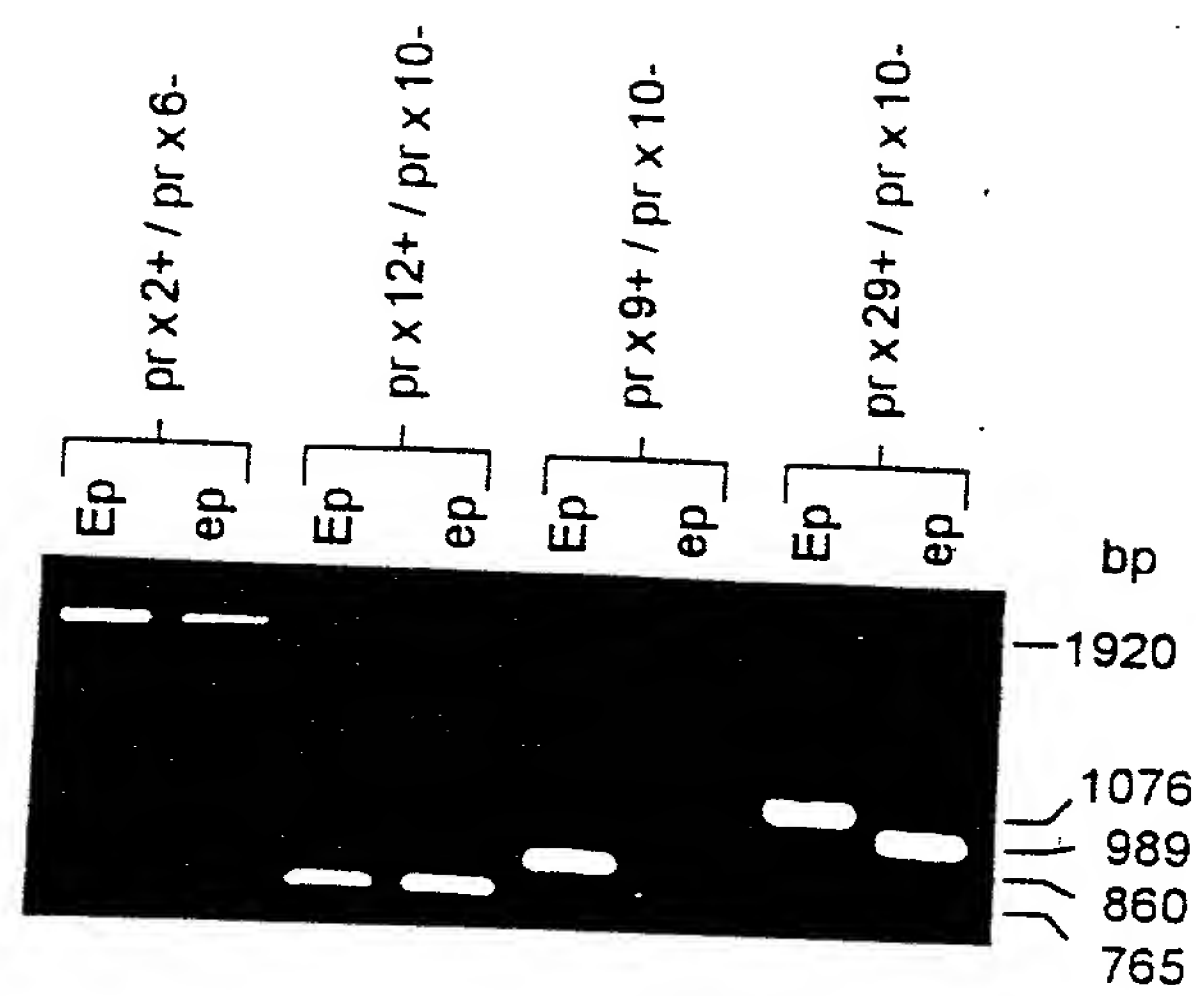


Diagram illustrating the OX347(Ep) gene construct and its expression in OX312(ep) cells. The top part shows a linear map of the OX347(Ep) construct, which is 17 kb long. It contains a BamHI site at the left end, an XbaI site, and another BamHI site at the right end. The bottom part shows a linear map of the OX312(ep) construct, which is 3.3 kb long. It contains a SacI site. Dotted lines indicate the homologous regions between the two constructs. Below the maps, the DNA sequences are shown: OX312(ep)...TAAAATCATAT...CAGCTTACTCC... and OX347(Ep)...TAAAATCATATTAAGT...ATGCTCAGCTTACTCC... The positions -10 and +79 are marked on the OX347(Ep) sequence.

FIGURE 6



The figure consists of two autoradiographs of agarose gels. The top gel is labeled 'P' and 'F2' with lanes M, -, +, +, +, -, +, -, +, -, +, -, +, +, -, +, +, M. The bottom gel is labeled 'F2' with lanes M, +, +, +, +, -, +, +, +, +, +, +, -, +, +, +, M. Molecular weight markers are indicated on the right at 1.5, 0.93, and 0.56 kb.

[illegible]

FIGURE 8

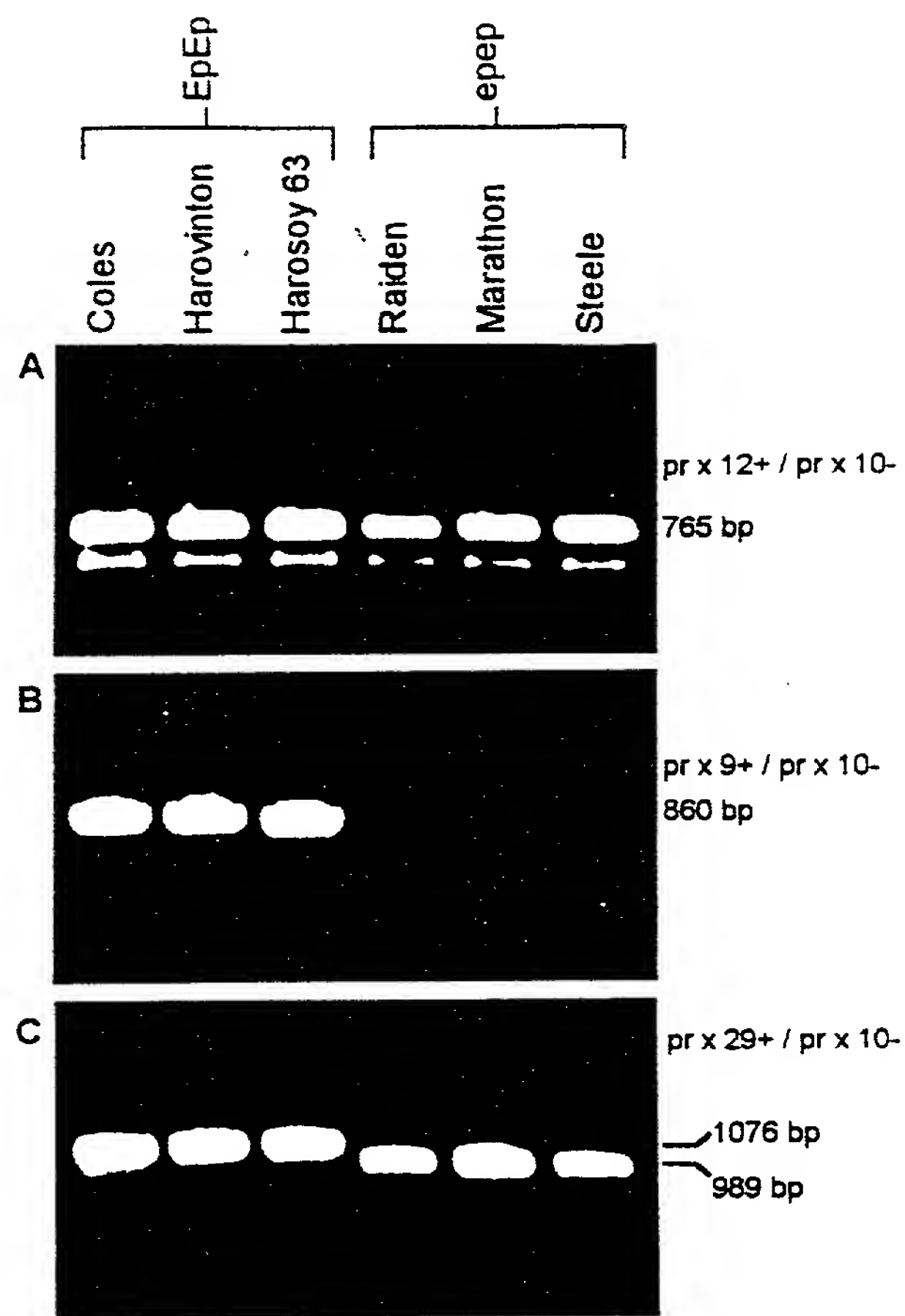
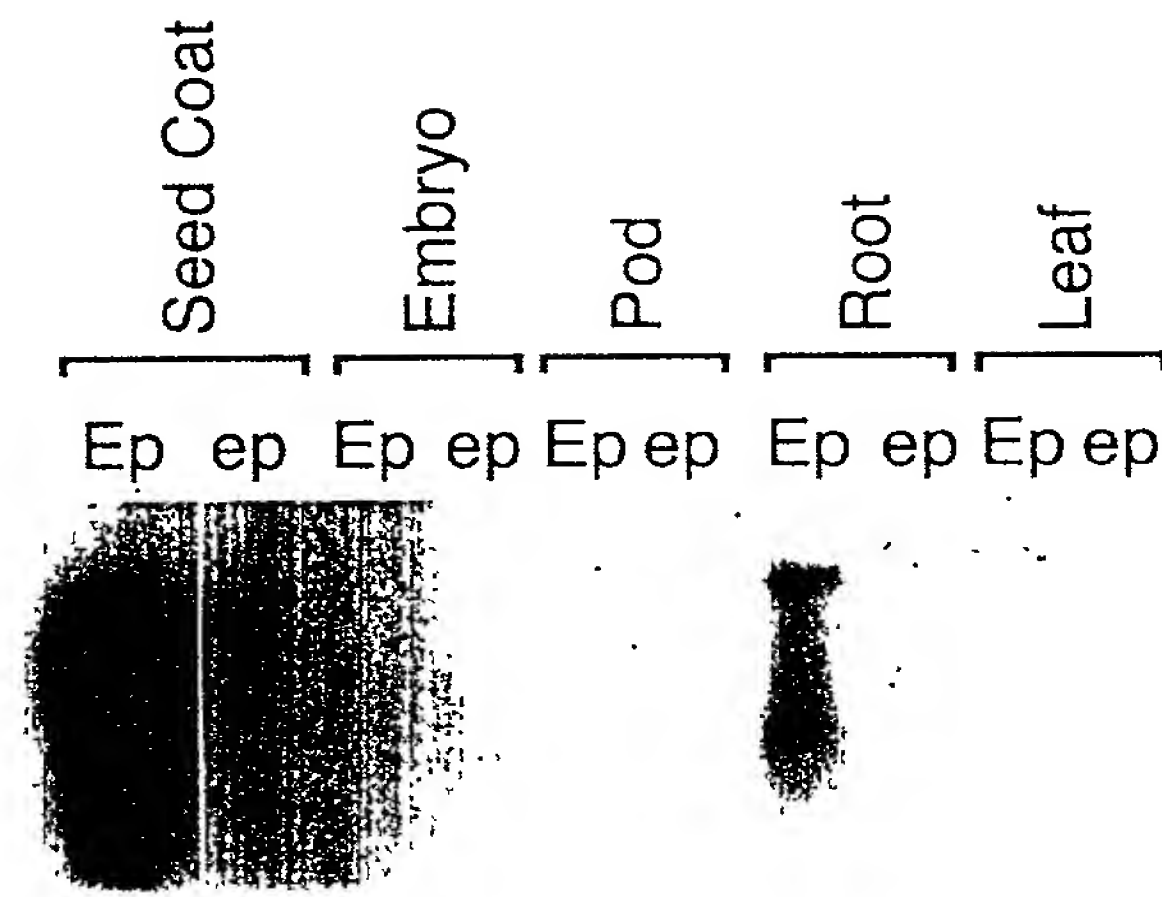


FIGURE 9

A



B

